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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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ANA CATARINA LEITE PEREIRA

Tese de Doutoramento em Ciências Biomédicas

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MESENCHYMAL STEM/STROMAL CELLS RECRUITMENT FOR INTERVERTEBRAL DISC REGENERATION

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Article 2

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Cunha, C., Almeida, C. R., Almeida, M. I., Silva, A. M., Molinos, M., Lamas, S., **Pereira, C.L**., Teixeira, G.Q., Monteiro, A.T., Santos, S.G., Gonçalves, R.M. (2016). Systemic delivery of bone marrow mesenchymal stem cells for in situ intervertebral disc regeneration. Stem Cells Translational Medicine, doi:10.5966/sctm.2016-0033.

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Science is not only a disciple of reason but, also,
one of romance and passion.

Stephen Hawking

Table of Contents

List of Abbreviations

A2M	Alpha-2-macroglobulin
AC	Articular cartilage
AD	Adipose Derived
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
AF	Annulus fibrousus
Agg	Aggrecan
ANXA3	Anexin A3
BASP1	Brain Abundant Membrane Attached Signal Protein 1
BM	Bone marrow
BMPs	Bone morphogenic proteins
C+hMSCs	Cavity + hMSCs group
CA	Carbonic anhydrase
CD	Cluster of differentiation
CDH2/NCAD	N-Cadherin
CEP	Cartilaginous endplate
CFU-F	Fibroblast colony-forming units
CILP	Cartilage Intermediate layer protein
Col Type	Collagen Type
COMP	Cartilage oligomeric matrix protein
COX	Cyclooxygenase
CS	Chondroitin sulphate
CTGF	Connective tissue growth factor
DCs	Dendritic Cells
DMMB	1,9-Dimethyl-Methylene Blue zinc chloride double salt
DNA	Deoxyribonucleic acid
DSC2	Desmocollin-2
ECM	Extracellular matrix
EGF	Epidermal growth factor
EPCs	Endothelial progenitor cells
ERK	Extracellular signal–regulated kinase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor

FOXF1	FORKHEAD BOX F1
G-CSF	GRANULOCYTE COLONY-STIMULATING FACTOR
GAG	GLYCOSAMINOGLYCANS
GD2	DISIALOGANGLIOSIDE
GDF-5	GROWTH AND DIFFERENTIATION FACTOR 5
GFP	GREEN FLUORESCENT PROTEIN
GLUT-1	GLUCOSE TRANSPORTER-1
GM CSF	GRANULOCYTE MACROPHAGE COLONY-STIMULATING FACTOR
GPC3	GLYPICAN 3
H&E	HEMATOXYLIN/EOSIN
HA	HYALURONIC ACID
HA-pNIPAM	THERMOREVERSIBLE HYALURONAN-POLY(N-ISOPROPYLACRYLAMIDE)
HAP	THERMOREVERSIBLE HYALURONAN-POLY(N-ISOPROPYLACRYLAMIDE)
HBB	HEMOGLOBIN B-CHAIN
HGF	HEPATOCYTE GROWTH FACTOR
HIF-1/2	STABILIZED HYPOXIA INDUCIBLE FACTOR 1/2
hMSC	HUMAN MESENCHYMAL STEM CELLS
HSCs	HEMATOPOIETIC STEM CELLS
IF	IMMUNOFLUORESCENSE
IGF-1	INSULIN-LIKE GROWTH FACTOR
IGF-1sR	INSULIN-LIKE GROWTH FACTOR 1 RECEPTOR
IGFBP	INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN
IHC	IMMUNOHISTOCHEMISTRY
IVD	INTERVERTEBRAL DISC
KRT	CYTOKERATIN
KS	KERATIN SULPHATE
LAC-Z	ß-GALACTOSIDASE
LBP	LOW BACK PAIN
MCP-1	MONOCYTE CHEMOATTRACTANT PROTEIN-1
MIP-1	MACROPHAGE INFLAMMATORY PROTEIN-1
MMP	METALLOPROTEINASE
MRI	MAGNETIC RESONACE IMAGING
MSC	MESENCHYMAL STEM CELLS
MT-1-MMP	MEMBRANE TYPE-1 MATRIX METALLOPROTEINASE
NCAM-1	NEURAL CELL ADHESION MOLECULE 1
NP	NUCLEOUS PULPOSUS

OP-1	OSTEOGENIC PROTEIN-1
OVO-2	OVOSTATIN
PAX-1	PAIRED BOX-1
PBS	PHOSPHATE-BUFFERED SALINE SOLUTION
PDGF	PLATELET-DERIVED GROWTH FACTOR
PDGF-R	PLATELET-DERIVED GROWTH FACTOR RECEPTOR
PEG	POLYETHYLENE GLYCOL
PLGF	PLACENTAL GROWTH FACTOR
PRP	PLATELET-RICH PLASMA
PTMC	POLY(LACTIDE-CO-TRIMETHYLENE CARBONATE)
PU	POLYURETHANE
RFU	RELATIVE FLUORESCENCE UNIT
RT-PCR	REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION
SCF	STEM CELL FACTOR
SDF-1	STROMAL CELL DERIVED FACTOR-1
SHH	SONIC HEDGEHOG
SNAP25	SYNAPTOSOMAL-ASSOCIATED PROTEIN 25
SO-FG	SAFRANIN-O FAST GREEN
SOSTDC1	SCLEROSTIN DOMAIN CONTAINING 1
SOX9	(SEX DETERMINING REGION Y)-BOX 9
TEM	TRANSMISSION ELECTRON MICROSCOPY
TGF-B	TRANSFORMING GROWTH FACTOR-B
TIE	TYROSINE-PROTEIN KINASE RECEPTOR
TIMP	TISSUE INHIBITORS OF METALLOPROTEINASES
TNMD	TENOMODULIN
VCAM-1	VASCULAR CELL ADHESION PROTEIN 1
VCAN	VERSICAN
VDR	VITAMIN D RECEPTOR
VLA-4	VERY LATE ANTIGEN-4
WB	WESTERN BLOT

Abstract

The intervertebral disc (IVD) is the largest avascular organ in the human body that undergoes a profound degeneration early in life. One of the hallmarks of IVD degeneration is the pronounced cell death and senescence, which added to the fact of being a non-vascularized organ results in a poorer chance of self-repair. IVD repair/regeneration is the ultimate challenge for spine researchers.

In this doctoral dissertation, the design of a new strategy for IVD regeneration was pursued, based on the repopulation of the degenerative IVD with viable cells that would replenish the lost matrix and stimulate endogenous cells. Cell transplantation to the IVD for that purpose has been widely investigated. However, here, we aimed to circumvent cell transplantation by recruiting cells towards the damaged/degenerating tissue. With that in mind, part of this work was dedicated to get proof of concept that human mesenchymal stem/stromal cells (hMSCs) could migrate from the cartilaginous endplate (CEP) towards a damaged disc/nucleotomized disc.

hMSCs not only migrated but also have contributed for extracellular matrix (ECM) remodeling by increasing the expression of collagen type II and aggrecan. These two key components of the IVD are known to provide to this organ its unique biomechanical properties and function. Driven by this achievement, we further explored the hypothesis of enhancing hMSCs recruitment towards the IVD to improve the disc repopulation upon damage/degeneration. For this, a thermoreversible chemoattractant hyaluronan (HA)-based hydrogel was developed (HAP), containing a well-known chemokine, stromal cell derived factor-1 (SDF-1). This delivery system was shown to be able to deliver, in a sustained manner, the chemokine both *in vitro* and in an IVD *ex vivo* model. Moreover, the presence of the HA-based hydrogel containing SDF-1 could significantly enhance the recruitment of hMSCs from the CEP, thus increasing cell population within the tissue. This effect appears to be weakened by cell donor's age and cells ability to respond to the chemoattractant gradient.

Finally, we intended to understand how to trigger a higher hMSCs recruitment using an SDF-1-delivery system, could accelerate and/or improve the hMSCs regenerative effect on matrix remodeling. Using an *ex vivo* IVD model, we demonstrated that a higher cell recruitment might result in a faster regenerative effect, namely, in what respects to an increased collagen type II synthesis.

Overall, the work performed under the scope of this thesis has contributed to new insights on cell migration to the IVD tissue, has supported the use of CEP as an alternative

route for cells migration in the IVD, and has further contributed to the development of a chemoattractant delivery system that can significantly enhance stem cell migration to the IVD and accelerate repair.

Together with recent findings describing the ability of discs to secrete themselves recruitment factors and the existence of stem cells niches in the IVD surroundings, we consider that the work herein described presents new perspectives for the development of innovative IVD regeneration approaches based on the stimulation of stem cell recruitment and endogenous repair.

Resumo

O disco intervertebral (IVD) é o maior órgão não-vascularizado no corpo humano que padece de uma profunda degeneração desde uma fase inicial da vida. Uma das principais características da degeneração do IVD é a exacerbada morte celular e senescência, que acrescentada ao facto de ser tratar de um órgão não-vascularizado culmina numa baixa capacidade de autorreparação. A reparação/regeneração do IVD é um dos maiores desafios para investigadores da área da coluna vertebral.

Esta dissertação de doutoramento, teve como objectivo o desenvolvimento de uma nova estratégia para a regeneração do IVD, com base no repovoamento do IVD degenerado com células viáveis, que possam produzir a matriz perdida, assim como estimular as células endógenas. O transplante de células para este fim tem sido amplamente investigado. No entanto, neste projeto, o nosso objetivo foi contornar o transplante celular, recrutando células em direção ao tecido danificado/degenerado. Com este objetivo em mente, parte deste trabalho foi dedicado a demonstrar que células mesenquimatosas estaminais/estromais humanas (hMSCs) poderiam migrar através da placa cartilagínea (CEP) até ao disco danificado/nucleotomizado.

Estas células (hMSCs), não só migraram como foram capazes de contribuir para a remodelação da matriz extracelular (ECM), através do aumento da produção de colagénio tipo II e agrecano. Ambos são componentes fundamentais da ECM, que providenciam a este tecido propriedades biomecânicas e funções únicas. No seguimento deste resultado, foi explorada a hipótese de se potenciar o recrutamento de hMSCs para o disco, aumentando desta forma o repovoamento do disco após lesão/degeneração. Para tal, foi desenvolvido um sistema quimiotático usando um hidrogel termoreversivel composto de ácido hialuronico (HAP) e contendo uma conhecida quimiocina, SDF-1 (stromal cell derived factor-1). Este sistema de libertação, demonstrou ser capaz de libertar de forma controlada a quimiocina, tanto em condições *in vitro* como num modelo *ex vivo* de disco. Além disso, a presença do HAP contendo SDF-1, foi capaz de aumentar significativamente o recrutamento de células da CEP para o disco, aumentando desta forma a população celular deste tecido. Este efeito parece, no entanto, ser debilitado pela idade do dador e a capacidade de as suas células responderem a um gradiente quimiotático.

Numa fase final, o nosso objetivo foi perceber como é que, aumentando o recrutamento de células para o disco usando um sistema de libertação com SDF-1, poderíamos acelerar ou aumentar o efeito regenerativo das hMSCs na remodelação da matriz.

Usando um modelo *ex vivo* de disco, demonstrámos que um maior recrutamento de células poderá resultar num efeito regenerativo mais rápido, nomeadamente no que diz respeito ao aumento da síntese de colagénio tipo II.

Globalmente, o trabalho realizado no âmbito desta tese contribuiu para novas perspetivas sobre a migração celular para o IVD, para o uso da CEP como uma via alternativa para a migração de células para o disco, assim como para o desenvolvimento de um sistema de libertação quimiotático que pode potenciar significativamente a migração de células estaminais para o IVD e acelerar a sua regeneração.

Juntamente com os recentes trabalhos que descrevem a capacidade de os discos produzirem fatores de recrutamento, e a existência de nichos de células progenitoras em tecidos vizinhos do IVD, consideramos que o trabalho aqui descrito abre novas perspetivas para o desenvolvimento de abordagens inovadoras para a regeneração do IVD, através do estimulo de recrutamento de células progenitoras para a reparação endógena do tecido.

CHAPTER I

CHAPTER I

GENERAL INTRODUCTION

INTRODUCTION

The intervertebral disc (IVD) is a fascinating organ that combines biology and physics in a perfect manner. Due to its avascular nature, the IVD undergoes an early degenerative process when compared to other tissues in the human body. This degenerative process is often recognized as the main contributor for low back pain and radicular leg pain (Kuslich et al. 1991), although, most of the degenerative discs being described as asymptomatic (Jensen et al. 1994, Boos et al. 1995, Videman et al. 2003). Back pain affects approximately 632 million people globally (Vos et al. 2012), and it is estimated that 84% of the population will suffer from low back pain at some point of their lives (Walker 2000). This represents a substantial socio-economic burden, as a consequence of the high costs of health care, diminished productivity and absenteeism from work (Martin et al. 2008). Multiple conditions can result in back pain; however, disc degeneration is accounted for 40% of the cases (Cheung et al. 2009) which represents a large percentage and therefore consolidates the relevance of studying its nature and the underlying causes of IVD degeneration.

Like other cartilaginous tissues, the IVD has been considered to have none or very poor self-repair capacity, demanding a need for development of novel strategies for its regeneration. The treatment and regeneration of degenerated IVD still represents a significant challenge in medical science, due to its nature and all the biomechanics involved in its functions. Biology and engineering, two complex sciences, have been pursuing, in the past years, the unravelling of the IVD nature, in an attempt to discover in cells and/or materials a perfect combination that closely resembles such a particular organ and might contribute to its regeneration.

PHISIOLOGY OF THE INTERVERTEBRAL DISC

The IVD is a critical organ in the spine, by providing the flexibility and the capacity to absorb biomechanical forces. The IVD structure corresponds to one-third of the total human spine length (Pattappa et al. 2012). Besides the 23 discs that compose the spine, the human *columna vertebralis* consists of 33 bony vertebras positioned in five different regions: cervical (7), thoracic (12), lumbar (5), sacrum and coccyx (9) (Figure 1). The different structures of the spine represent distinct functions. The vertebra main function is to provide rigidity and support the trunk and extremities, protect the spinal cord and *cauda equina*, anchor the *erector spinae* and other muscles, whereas the IVD provides the flexibility and movement (Devereaux 2007). Throughout evolution the spine adaptation represented a major advantage for humans and other primates by providing the adoption of the upright bipedal stance which conferred several gains, namely, in vision and in the development of other bones and structures in the human

body. The discs are articulating structures between the vertebral body, which allow movement (flexion, extension and rotation) and act as a shock-absorbing organ in the spine; lack of this capacity would result in a rigid vertebral column (Roberts et al. 2006b).

Macroscopically, the healthy adult IVD is composed of different and interrelated tissues, the central nucleus pulposus (NP), the surrounding annulus fibrosus (AF), and the cartilaginous endplates (CEP), which provides the connection to vertebral bodies (Figure 1).

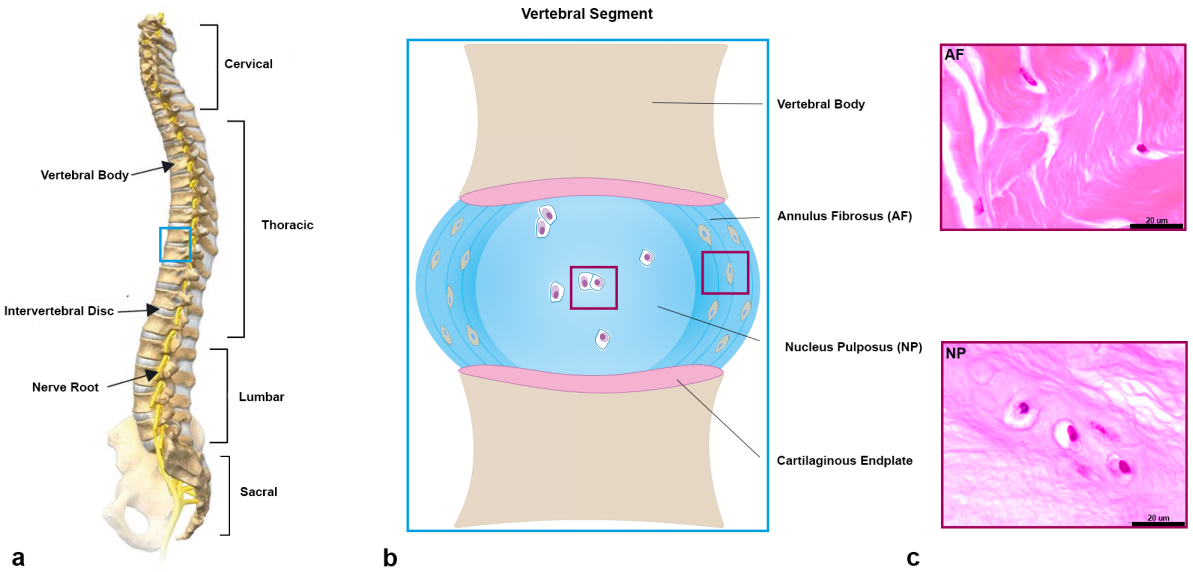

Figure 1 | Vertebral spine and IVD anatomy. **a |** Vertebral spine and its different regions: cervical, thoracic, lumbar and sacral. **b |** Vertebral segment: the IVD is located between two adjacent vertebras being interfaced by the cartilaginous endplate. The IVD is composed by the AF and the NP, that present cells with different morphologies. **c |** Representative images of the AF and NP cells within the tissue (scale bar: 20 μm).

The normal IVD comprises a large amount of extracellular matrix (ECM) interspersed by a small number of cells that account for approximately 1% of the total IVD volume. The IVD cells are believed to be constituted, at least, by two phenotypically distinct populations, depending on the IVD area, AF or NP. The IVD is composed by a dense matrix of collagens and proteoglycans, that together contribute to its particular biomechanical structure. While the collagen provides the disc its form and tensile strength, proteoglycans ensure the capacity to retain water and provide the stiffness, the resistance to compression and viscoelastic properties (Walker and Anderson 2004). The avascular nature of the IVD organ obliges that nutrition is provided by the blood supply at the disc's margins by diffusion of nutrients and metabolites towards the centre of the disc. IVD matrix composition, cells and nutrition will be further discussed in the following sections.

NUCLEUS PULPOSUS

COMPOSITION & FUNCTION

The NP is a complex and heterogeneous structure composed of randomly organized collagen fibbers embedded in a highly hydrated gel-like matrix rich in proteoglycans (Pattappa et al. 2012). The NP is predominantly composed of water (70-90%, depending on the age), proteoglycans (~50% of the dry weight) and collagen type II (col type II) (~20% of the dry weight) (Buckwalter 1995).

The proteoglycans hold a key role in the IVD biological function, by providing the capacity to retain water generating hydrostatic pressure within the NP, and contribute for the hydrodynamic and viscoelastic properties of the disc. Their composition differs from glycoproteins, by the replacement of their core protein with sulphated glycosaminoglycan (GAG) chains (Yanagishita 1993). Aggrecan (agg) is a proteoglycan from the keratin sulphate (KS)/chondroitin sulphate (CS) family and one of the most abundant proteoglycans in the NP tissue. It consists in a core protein containing KS and CS chains, interacting with hyaluronic acid filaments. These negatively charged chains enable the disc to remain highly hydrated and resist compressive loading in the spine, causing the disc to swell and keep the vertebrae apart (Melrose et al. 2001).

The collagen network found in the NP is similar to that found in articular cartilage (AC) (Eyre and Muir 1976). The primary function of collagen fibres is to provide a rough 3D network to support cells and to provide a framework that confines the proteoglycans. Col type II is the most abundant col type in the NP region. Contrasting to what is observed in AC, where collagen fibbers are highly organized to support tension due to osmotic swelling (Ateshian et al. 2009), the Col type II fibbers in the NP are disposed randomly. In the NP, the osmotic hydrostatic pressure is supported axially by the CEPs and radially by the tensile stresses in the AF (Steffen et al. 1998).

CELLULARITY

The NP tissue derives from the endoderm, being a remnant of the notochord (Roberts et al. 2006b). Depending on the species, the notochord cells may persist to adulthood. Many animal species retain the presence of these cells (Hunter et al. 2004), however, in humans the number of notochordal cells decreases drastically following birth, being non identifiable after four years (Roberts et al. 2006b). Still, the presence or loss of notochordal cells in humans remains debatable, as the presence of these cells was demonstrated in the human adult discs (Weiler et al. 2010). The notochordal cells, postnatal, are commonly identified in clusters, are

large in diameter (30-40 μm) and contain intracellular vacuoles of about 25% of the cell volume. In contrast, notochordal-derived mature NP cells are smaller round cells of about 10 μm in diameter and lack intracellular vacuoles. The cell density in the NP tissue is approximately $4x10^6$ cells/cm^3 (Pattappa et al. 2012).

Several studies contributed significantly to identify specific NP cell markers, and provided new insights in these cells development and metabolism. The identification of such markers is crucial for tissue engineering and regenerative strategies, namely, to monitor and trigger stem cell differentiation towards IVD cells. Still, a great part of the NP markers are not specific, and are very similar to those found in chondrocytes (Clouet et al. 2009), such as sex determining region Y (SRY)-box 9 (SOX-9) and col type II and agg markers, which were found to be expressed both in healthy and degenerative discs (Sive et al. 2002). Nonetheless, the ontogeny, the morphologic and physiological (matrix composition and biomechanical behaviour) differences observed between both NP cells and AC cells, suggests that despite the similarities, they represent two different phenotypes. Attempts to elucidate the phenotypic differences of NP and AC cells in different species cells are reported frequently in the literature. These studies provided a comprehensive list of NP markers in different species, but also emphasized the interspecies variations, which may have a crucial role in tissue/cell organisation, biomechanics and IVD microenvironment, highlighting the need for the identification of the human NP genetic signature, only a few markers were common between all the studied species. The NP cell makers described to date are summarized in Table 1. Despite several markers were identified only a few are considered to be primary markers, as established in 2014, by the Spine Research Interest Group meeting at the Annual Orthopaedic Research Society Meeting in New Orleans, which aimed to find a consensual definition of the NP cell phenotype. Together and based on the evaluation of different studies, they have recommended as NP phenotypic markers: the stabilized expression of hypoxia inducible factor-1α (HIF-1α), glucose transporter 1 (GLUT-1), agg/col type II ratio >20, sonic hedgehod marker (Shh), Brachyury, cytokeratins 18 and 19 (KRT18/19), carbonic anhydrase 12 (CA12), and CD24 (Risbud et al. 2015).

Table 1 | List of identified NP cell markers in the different species (adapted from (Rodrigues-Pinto et al. 2013, Risbud et al. 2015)

NP cell Marker	Species	Function in the IVD	Reference
Primary Markers			
Stabilized hypoxia inducible factor 1/2α (HIF-1/2α)	**H, S, R, M**	Transactivates many pro-survival genes in NP; absolutely necessary for post-natal NP cell survival	(Risbud et al. 2006, Agrawal et al. 2007, Agrawal et al. 2008)
Glucose Transporter-1 (GLUT-1)	**H, R**	Glucose transporter expressed in hypoxic tissues; expression controlled by HIF-1	(Rajpurohit et al. 2002, Richardson et al. 2008b)
Sonic hedgehog (Shh)	**H, M**	Signalling ligand necessary for post-natal function of NP cells.	(Dahia et al. 2012, Winkler et al. 2014)
Brachyury (T)	**H, B, D, M**	Transcription factor necessary for notochordal morphogenesis and patterning	(Minogue et al. 2010, Risbud and Shapiro 2011, Molinos et al. 2015b)
Aggrecan /collagen II ratio >20	**H, B, S, D, R, M**	High PG content maintains hydration to resist loads	(Mwale et al. 2004, Le Maitre et al. 2007c)
Carbonic anhydrase 12 (CA12)	**H, M**	Acid-base balance	(Lyons et al. 1991, Minogue et al. 2010, Power et al. 2011)
Cytokeratins 8, 18, and 19 (KRT8,18,19)	**H, B, D, R**	Cellular structural integrity and possibly signalling	(Lee et al. 2007) (Minogue et al. 2010) (Sakai et al. 2009)
CD24	**H, R, M**	Maintenance of NP homeostasis	(Fujita et al. 2005, Lee et al. 2007, Sakai et al. 2009, Minogue et al. 2010)
Secondary Markers			
Forkhead box f1 (FOXF1)	**B, H**	-	(Minogue et al. 2010)
Hemoglobin b-chain (HBB)	**H**	Storing oxygen under hypoxic conditions.	(Minogue et al. 2010, Rodrigues-Pinto et al. 2013)
Paired box-1 (PAX1)	**H**	-	(Minogue et al. 2010, Rodrigues-Pinto et al. 2013)
Ovostatin (OVO-2)	**H**	-	(Minogue et al. 2010)
Sclerostin domain containing 1 (SOSTDC1)	**B**	-	(Minogue et al. 2010)
N-Cadherin (CDH2)	**R, B**	N-Cad-mediated signalling regulates cell phenotype of juvenile NP cells	(Minogue et al. 2010, Hwang et al. 2015)
Synaptosomal-associated protein (SNAP25)	**B**	-	(Minogue et al. 2010)
α-2-Macroglobulin (A2M)	**H,D**	Proteases inhibitor	(Sakai et al. 2009, Rutges et al. 2010a, Wang et al. 2014a)
Neural cell adhesion molecule (NCAM1)	**D**	Embryogenesis and Development	(Sakai et al. 2009)
Desmocollin-2 (DSC2)	**D**	-	(Sakai et al. 2009)
Glypican 3 (GPC3)	**R**	Change in cell populations within the IVD (Apoptosis)	(Lee et al. 2007)
Anexin A3 (ANXA3)	**R**	-	(Lee et al. 2007)
Integrins α3, α6, b4	**H, P**	Cell Matrix Adhesion	(Nettles et al. 2004, Chen et al. 2009a)

H – Human; B – Bovine; P - Porcine; S – Sheep; D – Dog; R – Rat; M – Mouse.

ANNULUS FIBROSUS

COMPOSITION & FUNCTION

The AF tissue derives, as the spinal column, the vertebrae and the cartilaginous endplates, from the mesoderm (Roberts et al. 2006b). This tissue, is mainly composed of: water, about 60-80% depending on the age and the region; collagen (50-70% of the dry weight); proteoglycans (10-20% of the dry weight); and non-collagenous proteins as for instance elastin (~25% of the dry weight) (Pattappa et al. 2014). The AF consists of concentric layers of collagen type I (col type I), arranged in specific angles of 28º in the peripheral region and 44º in the central zone, with respect to the transverse plane of the disc. The space between the AF layers, the interlamellar septae, contains proteoglycan aggregates and other elements, which provides interlamellar cohesion. This highly organized structure provides AF an anisotropic behaviour, with the tensile, compressive, and shear properties differing in the axial, circumferential and radial directions, playing therefore a key role in the mechanical function of the IVD (Pattappa et al. 2012).

The AF can be further divided into an inner and an outer part, based on both structural and cellular differences. The inner part is less hydrated than the NP and the layers are more widely spaced compared to the outer AF. Concerning ECM components, the most abundant collagen type in the AF is the type I which increases from the inner part towards the outer annulus, still the type II is also present following an inverse distribution, making the transition to the NP (Bron et al. 2009a). Other proteins such as decorin and biglycan are mostly present in the outer AF, while collagen type X is mainly associated with the inner part and aged NP. Elastin, constitutes around 2% of the AF dry weight, having a role in the recoil properties of this tissue. Alterations in the ECM composition can severely affect the mechanical behaviour of the IVD. With aging, col type II is progressively replaced by col type I (Nerlich et al. 1998), leading to a shift in the mechanical properties from fluid pressurization to elastic deformation within the inner AF.

Nerves and blood vessels are both present in the AF to a limited degree in the healthy adult disc, restricted to the outer a few millimetres of the AF (Edgar 2007).

CELLULARITY

The AF has, comparatively to the NP, more than two times higher cellular density (around 9×10^6 cells/cm^3). Within AF, a higher cell density is found in the outer part when compared to the inner area. Morphologically, the AF cells are similar to fibroblasts, with a fusiform shape, thin and elongated in the outer AF whereas in the transition zone to the NP,

the inner AF, cells are round and resemble chondrocytes. These cells, derive from the mesenchyme and depending on their location they present their own morphology and synthesize distinct ECM. In the outer AF, cells are found isolated without any apparent physical/intercellular connection, and produced mainly col type I, while the cells of the inner part, in the interlamellar septae are more flattened, disc-shaped and produce col type II (Iu et al. 2014).

Different studies, some already mentioned in table 1, attempted to establish specific markers for NP and AF cells, and although some markers have been identified, in most of the cases, they are not exclusive of both cells. NP and AF cells share several phenotypic markers, varying only on their expression levels. In some particular cases, the expression profile of certain genes in the AF, when compared to the NP, was suggested as an AF marker. The suggested markers for AF cells are summarized in table 2.

Table 2 | List of AF cells suggested markers.

AF cell Marker	Species	Function in the IVD	Reference
Primary Markers			
Collagen type V	**Rb**	Form hybrids with col type I	(Clouet et al. 2009)
Tenomodulin (TNMD)	**H,B**	Avascularity of the tissue	(Minogue et al. 2010)
Relative (comparatively with NP cells) lower expression of:			
Cytokeratin 19 (KRT9)	**H**	Cellular structural integrity and possibly signalling	(Rutges et al. 2010a)
Neural cell adhesion molecule (NCAM1)	**H**	Embryogenesis and Development	(Rutges et al. 2010a)
α-2-Macroglobulin (A2M)	**H**	Proteases inhibitor	(Rutges et al. 2010a)
Desmocollin 2 (DSC2)	**H**	-	(Rutges et al. 2010a)
Relative (comparatively with NP cells) higher expression of:			
Glypican 3 (GPC3)		Change in cell populations within the IVD (Apoptosis)	(Iu et al. 2014)
Cartilage oligomeric matrix protein (COMP)	**H,R**	Collagen Fibril assembling	(Lee et al. 2007, Rutges et al. 2010a)
Versican (VCAN)	**H**	Hydration and viscoelasticity	(Hasegawa et al. 2007, Minogue et al. 2010)
Brain abundant: membrane attached signal protein 1 (BASP1)	**H**		(Minogue et al. 2010)

H – Human; B – Bovine; P - Porcine; S – Sheep; D – Dog; Rb – Rabbit; R – Rat; M – Mouse;

CARTILAGINOUS ENDPLATES

COMPOSITION & FUNCTION

The CEP is an osseous/cartilaginous tissue linking the IVD to its adjacent vertebras (Figure 1). At the time of birth, the human CEPs account to approximately 50% of the IVD space, when compared to adulthood, which is around 5%, and has large vascular channels passing through them. Following birth, these vascular channels are filled with extracellular matrix and no vascular vessels remain by the first decade of life, turning the IVD in the, so called, largest avascular organ in the body in adulthood (Roberts et al. 2006b). In adults, the CEP is a thin layer (0,1-1 mm thick) of hyaline cartilage with calcified cartilage adjoining the bone. Similar to the other IVD tissues (AF and NP), the CEP is also mainly composed of water (70-80% depending on the age) followed by col type II and proteoglycans (Pattappa et al. 2012).

The CEP main functions are structural and mechanical, though this tissue also works as a semi-permeable barrier by providing the nutrient supply to the avascular NP. Mechanically, the CEP is responsible for the distribution of the IVD compressive loading to the vertebral body. This capacity is assured by the CEP composition (balance of collagen, proteoglycans and water) and may vary accordingly to the phase of matrix turnover: growth, maturation, aging and degeneration, as proposed in the study of Antoniou et al. (Antoniou et al. 1996). Proteoglycan molecules have a pivotal role in the control of solute transport through the disc, and the depletion of proteoglycans from the CEP is associated with loss of proteoglycans from the NP (Roberts et al. 1996). Regarding the nutrients supply, the diffusion distance from the blood supply in the CEP to the cells in the NP central part can reach 8 mm (Benneker et al. 2005).

The failure of the CEP to perform these functions is hypothesized to accelerate disc degeneration, and although CEP permeability accounts for it, the molecules diffusion through the disc are also dependent on other factors such as the molecule size and ionic charge (Urban et al. 1977). Although some studies relate IVD degeneration with the decrease of CEP permeability or CEP calcification (Nachemson et al. 1970, Roberts et al. 1996, Benneker et al. 2005), others defend that its permeability and porosity increases with age, and that disc degeneration origin might correlate primarily to an altered cell function rather than the privation of disc nutrition through the endplate (Rodriguez et al. 2012).

CELLULARITY

The cells found in the CEP are very similar to those found in AC, regarding morphology (rounded) and density, $15x10^6$ cells/cm^3 (a higher density when compared to the AF and NP) (Maroudas et al. 1975). Contrarily to AF and NP cells, few studies have addressed CEP cells and their alterations with age and in pathologic circumstances. Similarly to the NP and AC cells, CEP-derived cells have a chondrocyte-like morphology and the ability of synthesise matrix components such as col type II, agg and hyaluronan. Liu et al., reported in 2001, for the first time, the existence of CEP-derived stem cells, which could be isolated, cultured and maintained several similarities (morphology, proliferation rate, phenotype and gene expression) with bone marrow-derived mesenchymal stem cells (BM-MSCs) (Liu et al. 2011), unveiling new perspectives on cell based candidates for IVD regeneration therapies.

CEP AS THE ROUTE OF IVD NUTRITION

The IVD, as the largest avascular organ in the human body, requires that nutrition is correctly supplied in order to retain its viability and biological function. The main supply routes of the IVD concentrate in its marginal areas such as the AF and the CEPs, through where essential nutrients such oxygen and glucose can diffuse to the whole tissue (Figure 2a). From the tissue, a reverse route is used for cleansing of metabolic waste products (Holm et al. 1981), creating a concentration gradient of nutrients/metabolites determined by the rates of nutrient supply/consumption by the cells (Figure 2b). Despite, in the first decade of life, the blood supply is provided through both CEP and AF, these blood vessels recede with age and in adulthood, CEP is the main route by which small solutes diffuse into the disc. The central part of the CEP presents more permeability, in comparison with the periphery, being the area with the highest diffusion (Maroudas et al. 1975). The nutrient supply occurs through the capillaries and nutrient canals present in the dense hyaline cartilage of the CEP, functioning as a selective permeable barrier to solutes diffusing based on their charge and size. Molecules diffusion in the matrix is also mediated by its composition, that consists mostly of collagen fibbers and the polyanionic proteoglycans, enhancing the entrance of cationic particles. Oxygen, water and amino acids easily diffuse due to their small size and neutral charge, while anionic and high molecular weight molecules are selectively excluded (Roberts et al. 1996).

Disc degeneration is closely associated with the disruption of the nutrient supply and can occur by three main reasons: shortage of blood supply itself, an impairment that can be caused by several disorders, such as, atherosclerosis of the abdominal aorta, thrombophilic and hypofibrinolytic diseases; external factors, commonly, exposure to vibration, smoking, and others that have a direct impact in the nutrient supply regulation; and, sclerosis of subchondral

bone or CEP calcification (Urban et al. 2004). Though CEP calcification can restrict the nutrient supply, to date it is still not clear, whether it represent a cause or an effect observation; calcification can lead to cell death due to the lack of nutrition, or CEP calcification can be caused by the biochemical and physical alterations of the disc during the degenerative process. Some of the signs of nutrient transport disturbance in the disc are reflected in their high levels of lactic acid and acidic pH (Roberts et al. 1996). Normal disc tissue have a mildly acidic pH of around 6.9-7.2, decreasing in degenerative conditions to values as low as 6.1 and contributing to matrix deterioration (Ohshima and Urban 1992). Low pH is as consequence of lactic acid accumulation, since the hypoxic nature of the organ requires the shift to an anaerobic metabolism of the cells (Urban et al. 2004).

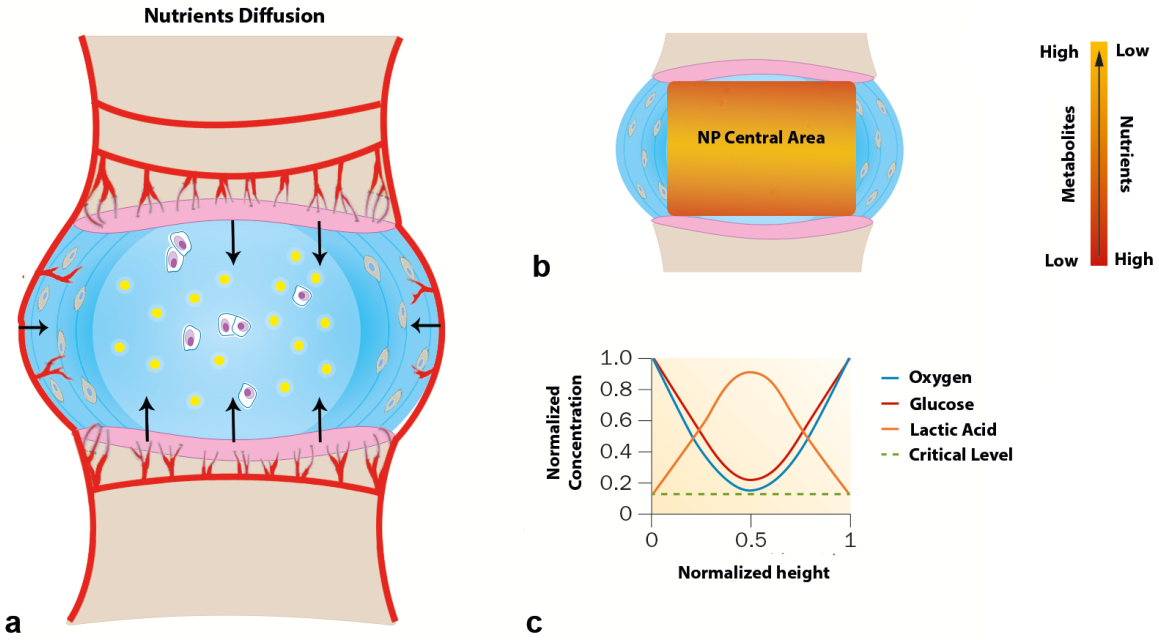

Figure 2 | Pathways of nutrient supply in a normal intervertebral disc. **a |** Cells of the avascular disc NP and inner AF are supplied by vertebral blood vessels. Capillaries penetrate the subchondral plate through marrow spaces and terminate in loops at the junction of the subchondral plate and CEP. **b |** Nutrients (e.g., oxygen and glucose) diffuse from the capillary bed through the CEP under gradient forces arising from metabolic demands of disc cells, while metabolic wastes (e.g. lactic acid) diffuses in the reverse direction. Cells of the outer AF are supplied by capillaries from blood vessels in the surrounding soft tissues that penetrate a few millimetres into the disc. The centre of the disc has the lowest levels of nutrients and highest concentration of metabolites. **c |** Schematic showing normalized concentration gradients of glucose, oxygen and lactic acid across the nucleus, endplate–endplate. Nutrient concentrations must remain above the critical levels to maintain cell viability and activity (Huang et al. 2014). (Image adapted from Huang et al. (Huang et al. 2014)).

PROGENITOR CELLS IN THE INTERVERTEBRAL DISC

Several findings supported, in the past years, the presence of progenitor stem cells in the IVD. The presence of calcifications, fibrocartilage-like tissue and nerve/blood vessels growth detected in pathological conditions, suggests that these cells originated from resident stem/progenitor cells (Freemont et al. 2002, Nerlich et al. 2007, Rutges et al. 2010b).

Different studies demonstrated the presence of an endogenous stem cell population within the IVD. Risbud et al., described a heterogeneous population of skeletal progenitor cells in human degenerated IVDs, expressing several characteristic proteins of MSCs (CD105, CD166, CD63, CD49a, CD90, CD73 and CD133) and negative for CD34. These cells were also able to differentiate into the typical osteogenic, chondrogenic and adipogenic lineages (Risbud et al. 2007, Blanco et al. 2010).

The presence of stem cells in the AF area was also suggested. Henriksson et al., identified stem cell markers in the AF border lo ligament zone (Henriksson et al. 2009a) and Feng et al. following AF cells isolation, demonstrated that these cells could express cell surface antigens associated with MSCs, such as CD29, CD49e, CD73, CD105, CD166, CD184 and Stro-1. AF-derived stem cells were also capable of differentiate into the different mesenchymal lineages (adipocytes, osteoblasts, chondrocytes, neurons and endothelial cells) (Feng et al. 2010). More recently, Sakai et al., identified populations of progenitor cells in the NP of both mice and humans. These cells were positive for tyrosine-protein kinase receptor ($Tie2^+$) and disialoganglioside 2 ($GD2^+$), with the capacity to form spheroid colonies and able to express both col type II and agg; they also presented differentiation capacity towards mesenchymal lineages. Interestingly, the frequency of $Tie2^+$ cells decreased with age and degeneration thus compromising the endogenous regenerative capacity (Sakai et al. 2012). Recently, Liu et al. identified a sub-population of cells derived from the human AF expressing typical surface antigen markers of MSCs, including CD29, CD44, and CD166, and capable of differentiate into chondrogenic and adipogenic lineages, suggesting that cells in the AF can be skeletal progenitor cells recruited under pathologic conditions, e.g., herniation, or progenitor cells from surrounding tissue with migration capacity towards the intervertebral disc under these circumstances (Liu et al. 2014).

These studies offered novel insights on the endogenous repair capacity of the IVD and raised some expectations for treatments aiming to stimulate endogenous healthy cells to revert the degenerative process.

INTERVERTEBRAL DISC DEGENERATION: CAUSES AND CONSEQUENCES

DISC DEGENERATION

In the early 70's, Schmorl and Junghanns defined the disc as the unique organ in the human body that undergoes a profound degeneration early in life (usually in the second decade) (Schmorl and Junghanns 1971). Disc degeneration is an age-related multifactorial process encompassing both genetic and environmental contributions. Although several environmental factors have been demonstrated to influence disc degeneration, such as, smoking, diabetes, infection, trauma, heavy lifting and vibration, the genetic component is recognized to play the predominant role in early disc degeneration and external factor susceptibility (Walker and Anderson 2004).

Disc degeneration is described as progressive process, entailing significant histomorphological and biomechanical changes as a consequence of an altered cell metabolism and an unbalanced matrix synthesis (Walker and Anderson 2004). These alterations result from a cascade of disrupting events which are believed to be triggered by impaired disc nutrition, followed by an accumulation of waste products and matrix degradation (Molinos et al. 2015a). Following this, the cellular microenvironment of the disc becomes progressively more hostile, characterized by an acidic pH and upregulation of several matrix degrading enzymes, and with the release of proinflammatory cytokines (Smith et al. 2011).

Cell viability in the IVD is dependent of a successful nutrition of the disc, but it is also linked to the normal aging process. Apoptosis was described to play an important role in disc degeneration, namely in older individuals (Gruber and Hanley 1998). Adding to this, several changes in terms of cell metabolism occur and have a profound impact in the synthesis of the main matrix components (col type II and agg); this loss of matrix was observed in discs from older individuals as reported in the study of Antoniou et al. in 1996 (Antoniou et al. 1996). The matrix loss observed in the course of the degenerative process, might also be related to the production of matrix degrading enzymes and other catabolic molecules, as well, as the presence of oxygen free radicals, nitric oxide, interleukins and prostaglandins.

The degeneration process is characterized by an elevation in levels of the inflammatory such as the cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-1 α/β (Le Maitre et al. 2005). These molecules are chemotactic to neutrophils, induce the expression of adhesion molecules on endothelial cells, can stimulate phagocytosis and the production of several other molecules that increase the rate of matrix breakdown (Shinmei et al. 1988, Risbud and Shapiro 2014). Among those are the metalloproteinases (MMPs), such as MMP-1, -3 and -9, that are present in degenerative discs (Kanemoto et al. 1996, Crean et al. 1997); aggrecanases (a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS-4/5)) (Roberts et al.

2000); and prostaglandin E_2 (PGE_2) (Kang et al. 1996). IL-6 can also be secreted by disc cells and its expression was shown to be elevated in herniated discs. This cytokine is believed to potentiate the catabolic action of both IL-1 and TNF-α (Studer et al. 2011). Other cytokines such as IL-17 and IFN-γ, are involved in disc inflammatory response, by recruiting immune cells to the injury (Risbud and Shapiro 2014).

Overall, these events result in an imbalance of catabolic and anabolic activities that contribute to tissue weakness and culminate in loss of the biological function (Figure 3).

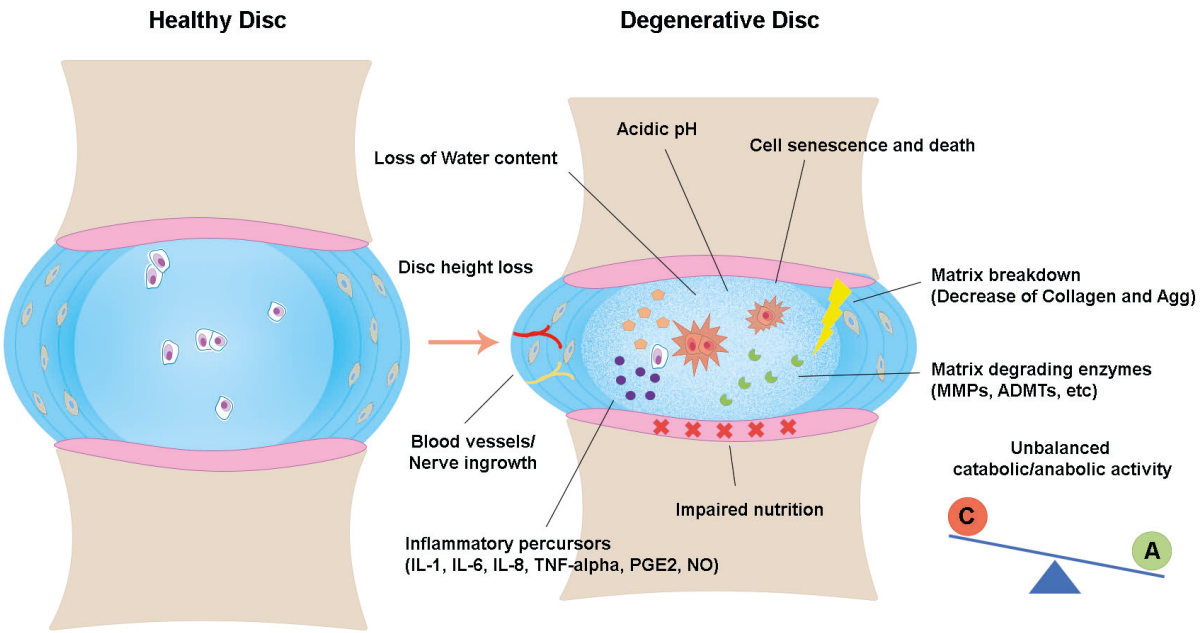

Figure 3 | Disc degenerative process: changes and involved molecules. Healthy disc versus degenerative disc. Impaired nutrition can trigger disc degeneration process. This process is characterized by the loss of water content and reduced, resultant from matrix breakdown and the hostile environment in the disc (inflammatory precursors release, matrix degrading enzymes and acidic pH), as well as an increased cell death and senescence that together contribute for an unbalanced catabolic/anabolic activity.

GENETIC FACTORS INVOLVED IN IVD DEGENERATION

The underlying cause of IVD degeneration resides on tissue weakening, occurring primarily due to genetic predisposition. A study, using a twins' cohort, was able to show that the heritability of disc degeneration was observed in about 29-54% of the elements studied, with the remaining corresponding to environmental influences (46-71%) varying at the spinal level (Battie et al. 2008); this represented a clear demonstration of the genetic background contribution in disc degeneration. The genetic factors associated with IVD degeneration have been studied in the past years in an attempt to shed some light regarding the underling mechanisms of disc degeneration, and to develop novel tools for early diagnosis and prevention. The genetic background of each individual, has an important role in disc aging and degeneration. In general, most of the characterized genes involved in the IVD pathologies are related to matrix structural molecules (Battie et al. 2004). Alterations in genes related with matrix components such as collagens and proteoglycans may exert their influence by weakening the matrix, leading to the initiation of the degenerative process. Other contributors may associate with alterations of tissue homeostasis by an unbalanced catabolic/anabolic activities occurring as a consequence of genetic alterations in catabolic genes, e.g., MMPs (Roberts et al. 2000), or genes related with proinflammatory cytokines, that might accelerate tissue degradation and compromise the disc structural integrity (Le Maitre et al. 2004, Le Maitre et al. 2007a). Numerous studies focused on several candidates genes and associations with disc degeneration have been found in more than 20 different genes, such as COL1A1, COL9A2 and COL9A3, COL11A2, agg (ACAN), vitamin D receptor (VDR) and MMP-3 but also in many other related proinflammatory cytokines and cells survival (Chan et al. 2006); some of those studies and genes are summarized on table 3.

The genetic defects reported might result in structural and functional changes of specific components of the disc ECM, compromising the mechanical properties and leaving it more susceptible to other external factors. These alterations may trigger metabolic changes further contributing to a higher incidence of disc degeneration in certain populations relatively to others. To date, most of the reported studies will still need additional validation due to the small cohorts used and lack of replication. Among the numerous genes identified, the most robust associations were detected in the VDR gene that were present cross three different populations (Videman et al. 1998, Kawaguchi et al. 2002b, Cheung et al. 2006); for the other genes, moderate conclusions should be drawn as they are dependent on additional confirmation.

Table 3 | Genes presenting genetic variants associated with IVD degeneration in humans.

GENE	GENE NAME	ASSOCIATION	REFERENCE
COL1A1	Collagen 1 alpha 1	Increase disc degeneration Alterations of ivd phenotype	(Pluijm et al. 2004, Tilkeridis et al. 2005, Videman et al. 2009)
COL9A1 **COL9A2** **COL9A3**	Collagen IX alpha 1 Collagen IX alpha 2 Collagen IX alpha 3	Increases/accelerates disc Degeneration and herniation	(Kimura et al. 1996, Boyd et al. 2008) (Annunen et al. 1999, Solovieva et al. 2006)
COL11A1	Collagen XI alpha 2	Disc herniation Disc bulges Signal intensity	(Noponen-Hietala et al. 2003, Solovieva et al. 2006)
IL-1A **IL-1B**	Interleukin 1 &1ß	Disc bulges	(Solovieva et al. 2004)
IL-6	Interleukin 6	Disc degeneration	(Kelempisioti et al. 2011)
ACAN	Aggrecan	Disc and cartilage degeneration	(Feng et al. 2016) (Kawaguchi et al. 1999)
MMP-3	Matrixmetalloproteinase 3	Disc degeneration	(Takahashi et al. 2001) (Vo et al. 2013)
VDR	Vitamin D receptor	Disc degeneration	(Videman et al. 1998, Videman et al. 2001, Kawaguchi et al. 2002b, Cheung et al. 2006).
MMP-2	Matrixmetalloproteinase 2	Disc degeneration	(Dong et al. 2007)
MMP-9	Matrixmetalloproteinase 9	Disc degeneration	(Sun et al. 2009)
IGF-1R	Insulin-like growth factor 1 receptor	Radiographic disc narrowing	(Urano et al. 2008)
CILP	Cartilage Intermediate layer protein	IVD degeneration, by the regulation of TGF-β signaling	(Seki et al. 2005, Seki et al. 2006, Virtanen et al. 2007)
TIMP1	Tissue inhibitor of metalloproteinase 1	Radiographic progression of lumbar disc degeneration	(Valdes et al. 2005)
COX2	Cyclooxygenase-2	Radiographic progression of lumbar disc degeneration.	(Valdes et al. 2005)
THSD2	Thrombospondin	Disc herniation	(Valdes et al. 2005, Hirose et al. 2008)

THE AGING PROCESS

IVD degeneration is part of the normal aging process. The disc healthy and healing capacity depends largely on its cell population and metabolite transport, both factors subject to changes with increasing age. Age related changes in both the CEP and IVD are reported to occur in humans in the first decade of life (Boos et al. 2002). However, before this time, mild microscopic degenerative features are observed at two years of age. These features include a decrease in NP cells proliferation and alterations in cell density and matrix degeneration in the CEP. These are believed to be correlated with the regression of blood vessels penetrating most of the AF tissue in early stages of life (Hassler 1969). As a consequence of the retreat of

blood vessels in the AF, a shortage of nutrient supply to this large avascular tissue occurs and culminates in decreased in cell density, promoting an early degenerative condition, contrarily to other tissues in the body. The disc's ability to heal is further reduced in late childhood by the loss of large metabolically active notochord cells from the NP (Adams et al. 2010).

Accompanying aging, it is also observed a progressive change in ECM components of the IVD tissue. Proteoglycans content decline with age, and the capacity of water retain is lost, occasioning a dramatic structural change in the disc. The disc becomes less hydrated (Antoniou et al. 1996) and the fluid pressure within it decreases, inducing the formation of radial disc bulges. As the disc ages, there is an increased deposition of coarse fibres of col type I in the inner AF and NP (Adams et al. 2010). Other progressive changes that occur in the IVD tissue with aging, were extensively described in the work of Boos et al. (Boos et al. 2002) and include an increased number of clefts and tears, the presence of granular material and neovascularization from the outer AF (Figure 3), as well as, cell proliferation, cluster formation and major cell death. Adding to this, there is a loss of differentiation between AF and NP areas. In the CEP, the aging process involves the thinning of the endplate, cracking, altered cell density, microfractures in the adjacent subchondral bone and bone sclerosis (Boos et al. 2002).

CONSEQUENCES OF IVD DEGENERATION

The disc-associated pathologies definition varies and are greatly dependent on the methodology used to study the disc. In the past years, these pathologies have been extensively characterized using advanced imaging techniques and diagnostics, namely, magnetic resonance imaging (MRI), the most common and non-invasive technique for IVD pathology diagnose. Along the years, based on the MRI image analysis and other techniques, it was possible to grade the severity of IVD degeneration, identify the presence of herniation and other bone-related pathologies and categorize different, but related, types of IVD degenerative conditions. It is often difficult to distinguish IVD pathologies from each other and the natural aging process; and to define whether the appearance of one predisposes to another.

Alterations to the disc health, results in dramatic changes in the alignment and mechanical milieu of the vertebral bodies, facet joints, spinal ligaments and muscles that eventually translate in pain. Besides the disc degeneration itself, there are other disc-related pathologies evolving from a compromised IVD and share a common symptom, pain. Among those, are the discogenic low back pain, disc herniation, spinal stenosis and/or spondylolisthesis (Table 4). A strong association of disc degeneration with low back pain (Lindblom 1948, Kelsey and White 1980, Lutz et al. 2003, Chou et al. 2011) was stablished

throughout the years. On the other hand, this relation is debatable due to the high non-symptomatic patients presenting disc degeneration; only 40% of low back pain cases have an obvious association with IVD as a pain source (Schwarzer et al. 1995, Cheung et al. 2009). A summary of the causes, symptoms and current treatments of disc-related pathologies can be found in table 4.

Table 4 | IVD pathologies.

PATHOLOGY	STRUCTURAL ALTERATIONS	Reference
DISCOGENIC PAIN	Abnormal micromotion instability • Starts with the circumferential fissuring of the outer AF, upon repetitive microtrauma and limited nutrition, Followed by delamination of AF layers and formation of radial tears. • Decrease in proteoglycans organization in the NP, loss of water content and osmotic pressure causes disc instability. Narrowing of the disc space. Inflammation: • Production of pro-inflammatory molecules (IL-1, TNF-α, RANTES/CCL5) • Nociceptive nerve fibre ingrowth	**(Yong-Hing and Kirkaldy-Willis 1983, Molinos et al. 2015a)**
SYMPTOMS	**Back Pain**	
TREATMENTS	Conservative (Physical therapy, rest, anti-inflammatory analgesia). Surgery.	
DISC HERNIATION .	Displacement of the IVD nuclear material beyond the normal contours of the outer AF. The histology of herniated disc varies accordingly to its degree of structural damage: • Disc Bulge • Protrusion (outer annular lamellae remain intact) • Extrusion (rupture of the annular lamellae) • Sequestration (herniation in completely detached from the body of the disc). Herniated discs are often more vascularized than normal intervertebral disc and have also an increase in matrix degrading enzymes such as MMP-8 and MMP-7, as well as an increase of inflammatory mediators such TNF-α and its stimulating gene TSG-6).	**(Roberts et al. 2005) (Fardon et al. 2001)**
SYMPTOMS	**Back Pain, Radicular leg pain**	
TREATMENTS	Conservative (Physical therapy, rest, anti-inflammatory analgesia. Surgery for Herniated disc removal	
LUMBAR STENOSIS	Narrowing of the neural canal* and neuroforaminal spaces of the lumbar spine. • The resultant encroachment and the narrowing of the neuroforamina from osteophyte formation, loss of disc space height, and hypertrophy of the ligamentum flavum can cause marked reduction in space available for the spinal nerve roots, leading to its compression and pain.	**(Lee et al. 2015)**
SYMPTOMS	**Intermittent low back pain without radiculopathy**. Radiculopathy or claudication with disease progression.	
TREATMENTS	NSAIDs analgesics, Physical therapy. Surgery	
DEGENERATIVE SPONDYLOLISTHESIS	Results from the facet joint degeneration and intervertebral disc degeneration. • Facet joints tend to enlarge in degenerative spondylolisthesis, which further encroaches into the spinal canal and can cause lateral recess stenosis and radicular symptoms.	**(Kalichman and Hunter 2008)**
SYMPTOMS	**Aching back pain, Claudication, Radiculopathy.**	
TREATMENTS	NSAIDs, and low-impact exercise programs. Surgery for Neural decompression with laminectomy and foraminotomy.	

INTERVERTEBRAL DISC DEGENERATION: TREATMENTS AND STRATEGIES

The current treatments for IVD degeneration and low back pain range from more conservative approaches to invasive surgical procedures. As previously mentioned (Table 4), conservative approaches are based on the use of pain killers and anti-inflammatory drugs and/or physical therapy while in worst case scenario the patient undergoes surgery for IVD removal. The gold standard surgical procedure for IVD degeneration or herniated discs is still spine fusion – no disc, no pain. Despite being the most commonly used procedure for patients who did not respond to the conservative treatments, this strategy may entail disadvantages as it alters the segmental motion of the spine, leading in some cases to further degeneration in adjacent discs (Etebar and Cahill 1999).

The total replacement of the IVD by non-biological prosthesis represents an alternative strategy to restore the segmental motion of the spine (Lee et al. 1991) and has been performed using mainly metallic, non-metallic or both types of materials. Still, the implantation of this types of prostheses can be a very traumatic surgery and the long-term results are limited due to device migration and limited life-spans as a consequence of the prosthesis wear, often requiring its removal or posterior fusions (Bao et al. 1996, Punt et al. 2008). IVD autograft and allograft strategies have also been described. Autograft implant has shown to be structurally sound, but the disc exhibited abnormal morphology and metabolic functioning (Frick et al. 1994), while allograft discs displayed evidence of progressive degeneration shortly following transplantation (Katsuura and Hukuda 1994, Luk et al. 2003).

All these strategies have a limited efficacy and do not produce predictable and reliable outcomes. They target the clinical symptom – pain, but are unable to do deeper address the underlying pathological problem in order to fully restore the tissue biological function.

TISSUE ENGINEERING APPROACHES

The treatments currently available for IVD degeneration do not take into consideration the biological and mechanical nature of this tissue. This flaw is an encouraging quest for the development of more bio/physiological and mechanically competent approaches focused on IVD preservation, repair, reinforcement and regeneration (Pereira et al. 2013). With the arising of therapies based on biomaterials and tissue engineering in other pathological/injury contexts, new strategies to treat the IVD have emerged, focused on biological solutions, by the introduction of functional cells and supporting biomaterials.

The several alterations in the IVD during degeneration, namely the loss of proteoglycans, impair the tissue water-retaining capacity, leading to the disc dehydration, prejudicing its biological function. Tissue engineering strategies aim to restore this function by

introducing shock-absorbing hydrogels or, in early degenerative stages, to delay or reverse the undergoing degenerative process using a biological stimulus, such as, matrix producing-cells and molecules that could stimulate the endogenous IVD cells to replenish the lost matrix. These strategies will be further discussed in the following section.

BIOMATERIALS

The use of biomaterials to repair/substitute the IVD focused in the replacement of IVD individual components, AF or NP, whereas few studies attempted to engineer a composite structure similar to the native tissue (Mizuno et al. 2004, Iatridis et al. 2013).

Concerning the AF, a wide range of materials have been studied aiming to mimic the tissue native biomechanical properties, which is very complex and with a unique structure. An AF substitute has been the biggest challenge; to date no material has fully met the tissue specifications (Iatridis et al. 2013). Promising scaffolds for AF tissue engineering using different materials, e.g., ateocollagen, alginate/collagen hybrid fibers and polycaprolactone, were already demonstrated to support AF cell adhesion, growth and ECM synthesis, but failed to address the mechanical functionality when evaluated *in vivo* (Sato et al. 2003b, Shao and Hunter 2007, Nerurkar et al. 2008, Pereira et al. 2013). Recently, AF repair was targeted from another perspective. Pirvu et al., developed a combined cellular scaffold using PTMC (poly(trimethylene carbonate) with MSCs and PU (poly(ester-urethane)) to locally repair the AF, offering immediate closure of the AF rupture and therefore reducing further degeneration (Pirvu et al. 2015). Although promising, the implantation of an AF repair strategy would still face several challenges, and would require the integration with other IVD tissues, the NP and/or the CEP. On the other hand, novel *in situ* closure techniques may offer clear solutions for the restoration of AF mechanical integrity (Bron et al. 2009a).

In the particular case of the NP repair, the main focus of most tissue engineering strategies is to develop materials that closely resemble the highly hydrated central part of the IVD. To meet this specification, hydrogels attracted particular attention due to their wide physical and chemical properties, closely resembling the characteristics of the gelly-like NP. The design of a NP-substitute must consider, not only its biodegradability and biocompatibility, but also other important criteria for its good performance. Properties such as: low viscosity, to allow a minimal invasive surgery, solidification after implantation (to avoid leaking of cells/gel), appropriate mechanical strength, resistance to degradation, ability to store large amounts of water, capacity to swell at various loadings and support cell growth and matrix production , are essential to take in account in the development of an adequate NP substitute (Pereira et al. 2013). Furthermore, the hydrogels should not provoke adverse biological response during degradation. Common materials include natural polymers, such as alginate, chitosan, agarose,

collagen and chondroitin sulphate, but although these materials are more closely to the ECM matrix composition, they lack the mechanical requirements of the NP. Contrarily, synthetic materials such as polylactic acid, polyglycolic acid and calcium phosphate can provide better mechanical properties, but fail in biocompatibility and swelling capacity. Finally, there are composite materials, which can combine both natural and synthetic materials to achieve both biological and mechanical goals (Yang et al. 2016).

Both cellular and acellular hydrogels have been developed for NP repair, some of those examples are summarized on table 5.

A great part of these studies has been focused in the *in vitro* assessment of the candidates, rather than its applicability locally in the IVD. Most of the studies using cellular hydrogels addressed mainly, cell viability and phenotype maintenance/differentiation of the cells (Baer et al. 2001, Richardson et al. 2008a, Chou and Nicoll 2009, Reza and Nicoll 2010, Jeong et al. 2014, Naqvi and Buckley 2015), while the few acellular ones, were particularly interested in the mechanical properties of the hydrogel (Wilke et al. 2006, Bron et al. 2009b, Omlor et al. 2012). Still, both studies by Wilke et al. and Omlor et al., tested, respectively, *ex vivo* (Wilke et al. 2006) and *in vivo* (Omlor et al. 2012) different hydrogels and confirmed how challenging it is to retain any NP substitute in the IVD. This reinforced the importance of focusing in the annular damage and new techniques for its repair, to assure disc integrity and the success of any IVD therapy based on materials injection in the NP.

Table 5 | Cellular and acellular hydrogels for NP substitution.

Material	Model	Cells	Cell Behavior	Mechanical behavior	Reference
Alginate					
Alginate hydrogels	*In vitro*	AF Cells (porcine)	Cells phenotype was maintained	↓ shear stress	(Baer et al. 2001)
Alginate hydrohel	*In vitro*	MSCs NP cells (Porcine)	↑GAG synthesis ↑Collagen accumulation	-	(Naqvi and Buckley 2015)
Calcium crosslinked alginate	*In vitro*	NP Cell (Human)	↑Cells viability in hydrogels with lowest % of modification	-	(Chou and Nicoll 2009)
Chitosan					
Chitosan hydrogel	*In vitro*	MSCs NP cells (Porcine)	↑GAG synthesis ↑Collagen accumulation	-	(Naqvi and Buckley 2015)
Chitosan Glycerophosphate	*In vitro*	MSCs (Human)	↑MSCs differentiation towards a NP phenotype	-	(Richardson et al. 2008a)
Collagen					
Collagen scaffolds	*In vitro*	-	-	↓ shear stress	(Bron et al. 2009b)
Collagen type I	*Ex vivo*	(Bovine)	↑Disc height	↑stability of flexion/extension movements *material extrusion	(Wilke et al. 2006)
Atelocollagen	*In vivo*	MSCs (Rabbit)	↑Support MSCs proliferation ↑Matrix synthesis ↑MSC diferentation ↓Disc degeneration	-	(Sakai et al. 2003)
Atelocollagen Type II	*In vitro*	NP Cells (Bovine)	↑Support NP cells Viability and phenotype	Similar composition to the NP ↑Scaffold volume mantained ↑injectability	(Halloran et al. 2008)
Hyaluronan					
Fibrinogen and hyaluronic acid conjugated hydrogels	*Ex vivo*	NP Cells (Bovine)	↑GAG synthesis ↑Disc height	↑Disc compreenssive stiffness	(Li et al. 2014b)
Hyaluronan/ Collagen hydrogel	*In vivo*	(Porcine)	Gene Expression: ↑ Col1, ↑Col2, ↑Decorin, ↑MMP13, ↑MMP3	↓ Disc height ↓MRIs signal intensity	(Omlor et al. 2012)
Hyaluronic Acid-Poly(ethylene glycol)	*In vitro*	NP/AF Cells (Porcine)	Support NP and AF cell culture	-	(Jeong et al. 2014)
Celulose					
Carboxymethylcellulose	*In vitro*	NP Cells (Bovine)	↑Pericelular deposition of proteoglycans	-	(Reza and Nicoll 2010)
Methacrylate					
Photo-crosslinked methacrylated gellan gum	*In vitro*	IVD Cells (Human)	Support IVD cells culture/viability	↑injectability ↑Mechanical properties in cellular hydrogels	(Silva-Correia et al. 2013)

GAGs – Glycosaminoglycans, Col1 – Collagen type I; Col2 – Collagen type II; MRI – Magnetic Ressonace imaging

GENE THERAPY AND GROWTH FACTORS

Several genes and growth factors are known to be involved in the anabolic and catabolic processes regulating matrix homeostasis in the IVD. Therapies able to delivery *in situ,* either, growth factors or nucleic acids, that could be applied to stimulate the disc endogenous repair capacity, are of great interest.

Gene therapy aims to modify the pattern of gene expression, ultimately resulting in an *in situ* sustained production of certain proteins. In the context of IVD regeneration, the use of gene therapy is, relatively to other diseases, poorly explored. One of the first studies using gene therapy was published by Wehling et al. in 1997, and used chondrocyte cells isolated from bovine CEPs, that were genetically modified and cultured *in vitro* (Wehling et al. 1997). The bacterial beta-galactosidase (LacZ) gene and the complementary DNA (DNA copy of the mRNA) of the human IL-1 receptor antagonist were introduced into the isolated cells by retrovirus mediated gene transfer. The treatment resulted in 1% of beta-galactosidase positive cells and in the production of IL -1 receptor antagonist protein (Wehling et al. 1997).

Following this, many studies in animal and human cells concerned the use of reporter genes, such as the encoding beta-galactosidase gene, green fluorescent protein (GFP) and luciferase, delivered via viral and non-viral methods, and explored whether the gene insertion would affect cells phenotype. Anti-inflammatory or anti-degeneration genes were also explored for the treatment of IVD degeneration. IL-1 receptor (IL-1Ra) gene insertion into NP and AF cells was demonstrated to impair matrix degradation (Le Maitre et al. 2007b). The gene delivery of tissue inhibitors of metalloproteinases-1 (TIMP-1) resulted in an increased measure of proteoglycans in cultured degenerated disc cells (Wallach et al. 2003). Seki et al. transfected rabbit NP cells with siRNA oligonucleotides specific for ADAMTS-5, and with the silencing of the gene, NP tissue degradation was suppressed (Seki et al. 2009).

Aiming to stimulate tissue regeneration, anabolic genes have also been targeted by gene therapy in IVD. Growth and differentiation factor-5 (GDF-5) (Cui et al. 2008), bone morphogenic proteins (BMPs) (Wang et al. 2011), transforming growth factor-β (TGF-β) (Lee et al. 2001), lim mineralization protein-1 (LMP-1) (Yoon et al. 2004) and Sox-9 (Zhang et al. 2009, Ren et al. 2015) gene therapy has been used to stimulate matrix synthesis, namely proteoglycans and collagen. In spite of the promising results, there are still several concerns regarding the safety of gene therapy strategies for IVD regeneration, as discussed in the works of Wallach and Levicoff (Wallach et al. 2006, Levicoff et al. 2008). Side effects of this therapy are related to dosage, transgene products or the vector choice, still both authors defend that the beneficial effects of this therapy outpace the risk. The application of this technology in clinical trials requires additional research to determine precise gene dosage and the

appropriate/accurate methods for delivery, especially in the disc due its proximity to vital neurovascular tissues.

The direct use of anabolic molecules – growth factors, rather than their gene expression, also became very popular. Growth factors are polypeptides that bind cell membranes by means of specific receptors. They are key players in any biological system but also on the IVD homeostasis, by regulating the metabolism within the disc and the production of matrix by the cells (Masuda and An 2004). TGF-β, is known as one of the protagonists growth factors in the IVD, by its significant role in the synthesis of collagen and proteoglycans (Konttinen et al. 1999). In the past years, there was a wide research on the efficacy of growth factors therapy, both *in vitro* and *in vivo*. Multiple growth factors were identified as stimulators of IVD cells and of ECM production. These studies were reviewed in the work of Wang et al. and some of the findings are summarized in table 6. In general, the biostimulation with individual growth factors presented a positive effect on matrix synthesis and cell proliferation (Wang et al. 2015). Alternatively, the combination of two or more growth factors, may improve the outcomes. *In vivo* studies have also shown the feasibility of growth factors injection in the treatment of IVD degeneration. The treatment of degenerated murine caudal discs with GDF-5 and TGF-β, showed to slow or reverse the decline of cellularity and proteoglycans content (Walsh et al. 2004). In rabbit, GDF-5 was able to restore disc height and improve MRI and histological scores (Li et al. 2004)

Another promising bioactive substance possible to inject in the IVD is platelet-rich plasma (PRP). PRP is an enriched cocktail of growth factors that was demonstrated to present beneficial effects in terms of cell proliferation and matrix synthesis (upregulation of agg and collagen), both in AF and NP cells (Akeda et al. 2006, Pirvu et al. 2014) and in the IVD tissue, using an *ex vivo* organ culture system (Chen et al. 2009b). *In vivo*, the injection of PRP in different models supressed the degenerative process, increasing chondrocyte-like cell proliferation and upregulating matrix expression, contributing to the preservation of water content and disc height (Sawamura et al. 2009, Gullung et al. 2011, Obata et al. 2012). In humans, a recent randomized controlled study using autologous PRP in the treatment of symptomatic degenerative discs revealed significant improvements in function and pain, in a one-year follow-up (Tuakli-Wosornu et al. 2016).

Besides the positive effect of growth factors and PRP therapies, there are still important considerations to take into account when applying such a treatment. This type of therapy would preferably target early stages of IVD degeneration, stimulating the proliferation and matrix production of the remaining functional cells, thus contributing for delaying/reverting the IVD degenerative process. The growth factor by itself will not influence the biochemical/mechanical properties of the tissue but the cells; nonetheless the reduced number of cells in higher

degrees of degeneration might impair the success of this therapy. In such conditions, its combination with cell-based strategies might be necessary, to achieve a favourable outcome.

Table 6 | Growth factors described to have regenerative potential for degenerated IVDs (*in vitro* studies). Adapted from Wang et al. 2015 (Wang et al. 2015).

GF	Sp.	Cell/Tissue	Effect	Reference
TGF-β	C,R, Rb	IVD and AF/NP Cells	↑ECM synthesis and cell proliferation	(Thompson et al. 1991, Hayes and Ralphs 2011, Lee et al. 2012)
BMP-2	H, Rb	NP/AF cells	↑PG synthesis ↑expression of Agg ↑expression Col type I & II	(Kim et al. 2003, Gilbertson et al. 2008, Lee et al. 2012)
BMP-12	H	NP/AF cells	↑ECM synthesis	(Gilbertson et al. 2008)
OP-1	Rb, H	NP/AF cells	↑Total DNA, PG and Col synthesis ↑Matrix repair	(Masuda et al. 2003, Takegami et al. 2005, Imai et al. 2007)
GDF-5	B, M	NP/AF cells	↑PG and Col synthesis Dose-dependent upregulation Agg and Col genes	(Li et al. 2004, Chujo et al. 2006)
IGF-1	C, B, H, R	IVD and NP/AF cells	↑ECM synthesis and cell proliferation ↓ %Apoptotic cells	(Thompson et al. 1991, Gruber et al. 2000, Pratsinis and Kletsas 2007, Hayes and Ralphs 2011)
PDGF	H, B	NP/AF cells	↓ %Apoptotic cells ↑Proliferation	(Gruber et al. 2000)
VEGF	Ms	NP cells	↑NP survival	(Fujita et al. 2008)
bFGF	C,B	IVD and NP/AF cells	↑ECM synthesis ↑Cell proliferation	(Thompson et al. 1991, Pratsinis and Kletsas 2007)
EGF	C	IVD	↑Proliferation	(Thompson et al. 1991)
CTGF	Rh	NP cells	↑PG and Col synthesis	(Liu et al. 2010)
PRP	H, P, B	IVD and AF/NP cells	↑NP proliferation and diferenciation ↑ECM synthesis	(Akeda et al. 2006, Chen et al. 2006, Chen et al. 2009b, Pirvu et al. 2014)

GF – Growth factors; Sp. – Species; H – Human; Rh - Rhesus monkey; C – Canine; B – Bovine; P – Porcine; Rb – Rabbit; R – Rat; PG - proteoglycan, IGF-1 -insulin-like growth factor 1, PDGF - platelet-derived growth factor, bFGF - basic fibroblast growth factor, EGF - endothelial growth factor, OP-1 - osteogenic protein 1, CTGF - connective tissue growth factor.

Cell-based therapies

One of the critical hallmarks of the IVD degenerative process is the decrease in cell viability and function, with a substantial proportion of cells existing in a senescent state (Roberts et al. 2006a, Gruber et al. 2007). A supplementation of the disc with viable cells could

be beneficial for new matrix synthesis and activation of the endogenous cells through paracrine factors. In this line of research, cell-based therapies have been largely explored for IVD degeneration treatment. But although native disc cells would be the most preferable source of cells to adapt to the disc environment and produce the appropriate matrix, harvesting and culture of these cells may be very demanding. The access to health IVD cells is very limited and to collect these cells from damaged discs could compromise the whole therapy. Several studies in rabbit, rats and dogs, addressed the role of native IVD cells as a therapy for IVD regeneration. Generally, all these studies were able to show that IVD-derived cells could delay the degenerative process and in some cases, promote regeneration and increase col type II expression (Nishimura and Mochida 1998, Okuma et al. 2000, Nomura et al. 2001, Gruber et al. 2002, Ganey et al. 2003, Sato et al. 2003a, Watanabe et al. 2003, Iwashina et al. 2006, Ruan et al. 2010, Huang et al. 2011). Although promising, there are still some issues regarding the presence of cells with morphological features of notochord cells in these models, and in some studies, an increase in col type II but not of proteoglycans was reported, which is fundamental to achieve IVD hydration (Sakai and Andersson 2015). Two studies reported the use of committed cells for IVD regeneration, namely the transplantation of chondrocytes from AC, that were shown to survive upon injection in the IVD and to produce ECM, namely col type II (Gorensek et al. 2004, Acosta et al. 2011). Due to the aforementioned, the great majority of cell-based studies published to date for IVD degeneration treatment has been focused in the regenerative potential of stem/progenitor cells, namely Mesenchymal Stem cells – MSCs.

Mesenchymal Stem Cells – Definition and properties. MSCs have been, in the past years, in the lights of any cell-based therapy. These cells were first described as “colony-forming fibroblast” when discovered in the bone marrow stroma in the 60’s, and defined by their capacity to form new bone when transplanted to an ectopic site (Friedenstein et al. 1966). The name MSCs – “Mesenchymal Stem Cells” was later proposed by Caplan and Pittenger (Caplan 1991, Pittenger et al. 1999). These cells are characterized by their “stemness”, often demonstrated by their potential capacity to differentiate into three distinct lineages: adipocytes, osteoblasts and chondrocytes (Jiang et al. 2002). Whether they originate from bone marrow cells or primitive cells in tissues, MSCs have been successfully isolated and expanded in number from diverse tissues. Different sources of stem cells have been pointed including the bone marrow, adipose tissue, the synovium, skin, tooth pulp and the umbilical cord (da Silva Meirelles et al. 2006). MSCs are often described as plastic-adherent cells expressing CD29, CD51, CD73, CD90 and CD105, while negative for CD31, CD45 and other markers of the hematopoietic lineage (Dominici et al. 2006). Other makers such as GD2 (Martinez et al. 2007), CD271 (Battula et al. 2009), and CD106 (Yang et al. 2013) have also been linked to the function of tissue derived MSCs or their subpopulations.

MSCs display a number of key features that confer several advantages over other cell populations. These cells can be easily accessed and isolated through minimal invasive techniques; can be culture and expanded *in vitro*; and can be used for allogeneic transplantation due to its low immunogenicity that allows these cells to escape immune recognition (Oh et al. 2008). They are known to present immunomodulatory properties and the ability to migrate to sites of injury/inflammation (De Becker and Riet 2016). When in the tissue, MSCs are assumed to have a therapeutic effect either by cell replacement, through the differentiation into the resident cells, or cell "empowerment", by reactivation of resident cells, through the secretion of paracrine factors (Wang et al. 2014b) (Figure 4).

MSCs have the ability to commit to different lineages following a particular stimulus, i.e., in the presence of different bioactive factors, *in vitro* and *in vivo*. Chondrogenic differentiation occurs in the presence of TGF-β and dexamethasone; the differentiation into the osteogenic lineage, on the other hand, is stimulated by dexamethasone, 13-glycerol phosphate, and ascorbic acid, whereas the adipogenic requires the presence of dexamethasone, insulin, indomethacin and 1-methyl-3-isobutylxanthine (Pittenger et al. 1999).

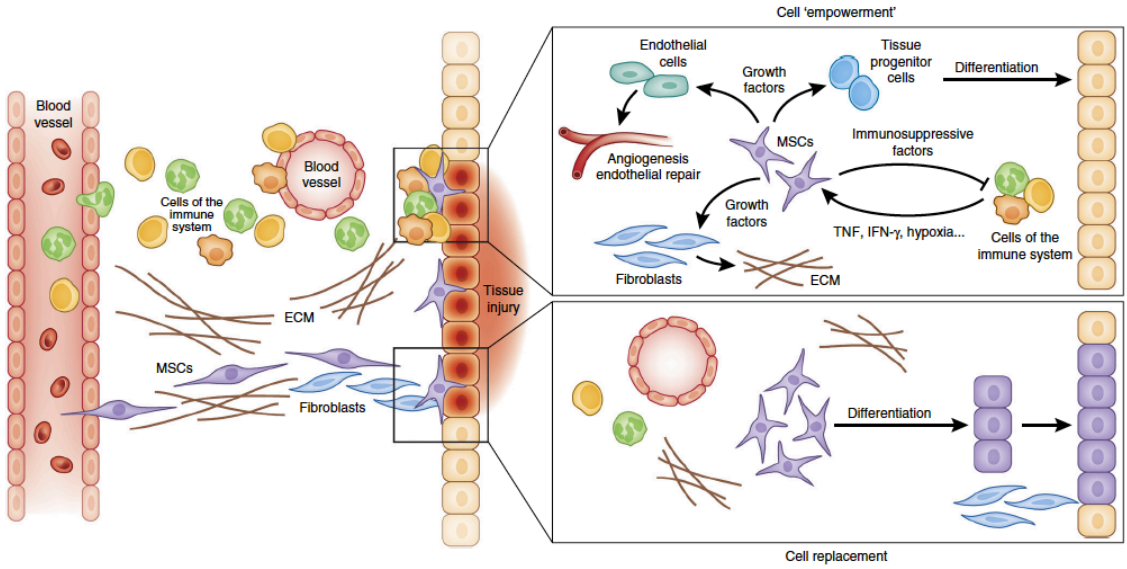

Figure 4 | Modes of MSC-based therapy: cell replacement versus cell 'empowerment'. After tissue damage, inflammation occurs and MSCs are mobilized to the injured tissue. Since they have multipotent differentiation potential, the MSCs recruited are believed to differentiate into functional cells to replace cells of the damaged tissue. However, in response to inflammatory cytokines, MSCs also help to prepare the microenvironment by producing immunoregulatory factors that modulate the progression of inflammation. MSCs also produce large amounts of growth factors which subsequently stimulate endothelial cells, fibroblasts and, most importantly, tissue progenitor cells *in situ*. The concerted action of these factors and cells facilitates tissue repair through angiogenesis, remodelling of the extracellular matrix (ECM) and the differentiation of tissue progenitor cells (Wang et al. 2014b).

For most of the time, the therapeutic effect of MSCs were thought to be exclusive of their multilineage differentiation capacity (Dominici et al. 2006). Nowadays, the increasing knowledge of the MSCs properties have revealed that their therapeutic effect can be mostly related with the secretion of growth factors and other molecules that act as immunomodulators in the tissue, despite of their poor engraftment and survival (Qian et al. 2008, Rose et al. 2008, Han et al. 2012, von Bahr et al. 2012). Recent studies using "MSCs secretome" have contributed further to the theory that MSCs exert their function through the release of immunosuppressive factors, cytokines, growth and differentiation factors (Katagiri et al. 2016, Teixeira et al. 2016a). Among those, are the IL-6, IL-10, TGF-β, PGE_2, HGF, FGF, PDGF, vascular endothelial growth factor (VEGF), IGF and nitric oxide (NO), that play a major role in the immunosuppressive capacity of MSCs (Zhao et al. 2016) by the inhibition or stimulation of immune cells. To date, the therapeutic effect of MSCs is considered to be largely dependent on their capacity to modulate tissue inflammation and homeostasis. The next section will examine in more detail the importance of these cells in IVD repair from *in vitro* studies and their application into different models of IVD degeneration.

STEM CELL-BASED THERAPIES FOR INTERVERTEBRAL DISC REGENERATION

In vitro studies. In vitro models have been, in the past years, the most useful tool to unravel the unique cellular biology of the disc. Monolayer co-culture of hMSCs in direct cell contact with both human NP cells from degenerated and non-degenerated discs, was first addressed in the work of Strassburg et al. (Strassburg et al. 2010). In both cases, degenerated or non-degenerated cells, hMSCs were able to differentiate to an NP-like phenotype by showing a significant upregulation of SOX9, Col type VI, agg and versican gene expression together with a simultaneous upregulation of cartilage-derived morphogenetic protein-1 (CDMP-1), TGF-β1, IGF-1 and connective tissue growth factor (CTGF). On the other hand, the presence of hMSCs had no effect on NP cells from normal discs, whereas stimulating NP cells from degenerated discs, enhances matrix-related genes expression and increased TGF-β and CDMP-1 gene expression, suggesting that MSCs can not only substitute NP cells, but also stimulate native cells to regain their function (Strassburg et al. 2010). Similar findings were described using human adipose-derived (AD) stem cells in direct co-culture with NP cells isolated from human degenerated discs, by a significant upregulation of multiple genes, proteins and NP markers, such as, SOX9, COL2A1, ACAN, COL6A2, FOXF1, PAX1, CA12, HBB, CDMP-1, TGF-β1, IGF-1, and CTGF. Further to this, degenerated NP cells presented an upregulation of COL2A1, ACAN, and COL6A2 (Sun et al. 2013). Activation of NP cells with MSCs was the focus of a study conducted by Watanabe and colleagues, where human NP cells and MSCs were co-cultured in direct cell-to-cell contact. Cell proliferation, DNA synthesis

and proteoglycans were significantly upregulated in the NP cells in the presence of the hMSCs (Watanabe et al. 2010). In a different setup, Vadalà et al. co-cultured both NP and MSCs in a 3D system. In this 3D environment, MSCs were shown to present a change in gene expression towards a chondrogenic lineage without cell fusion (Vadala et al. 2008). MSCs have also shown differentiation capacity to a NP-like phenotype, when cultured in a NP-derived matrix, up-regulating NP genes as col type II and glypican 3 (Yuan et al. 2013). In addition, MSCs differentiation into NP cells was shown to be also triggered by growth factors or hypoxia. Risbud et al. demonstrated that rat MSCs cultured in a media containing TGF-ß1 under hypoxia (2% O_2) could differentiate towards a phenotype consistent with that of the NP (mRNA upregulation of glucose transporter-3, MMP-2, col type II and type XI, and agg) (Risbud et al. 2004). MSCs differentiation have also been induced *in vitro* by media supplementation with GDF-5 or under indirect co-culture with NP cells (Stoyanov et al. 2011). Regarding NP cell markers expression profiling, KRT19 and CA12 were expressed to highest extent in the hypoxic GDF-5 groups (Stoyanov et al. 2011).

All these studies were essential in providing substantial knowledge regarding stem cells behaviour when in the presence of disc cells. Still, *in vitro* models lack the complexity of the IVD native tissue and therefore, the results obtained should be carefully considered and limited conclusions draw. Moreover, these models are also inadequate to assess the long-term effects of treatment.

Ex vivo studies. Ex vivo models are important alternatives to *in vivo* studies combining the control of *in vitro* experiments with a closer representation of the physiological environment of the tissue by maintaining the cells in their highly-specialized ECM. In IVD field, one of the most common explant model is the bovine caudal disc, by its similarities with human discs in what concerns to size and *in vivo* pressure, biochemical composition, cell density and a cell population lacking notochordal cells (Gantenbein et al. 2015). Other explanted discs from different animals, such as, rabbit and porcine, and human discs, have been reported, being the latter more recently described (Haschtmann et al. 2006, Chen et al. 2009b, Gawri et al. 2011, Peroglio et al. 2013, Walter et al. 2014). These models used isolated healthy discs that undergo induced degeneration *ex vivo* using different methodologies. Enzymatically digestion of the NP, by injection of enzymes such as papain, trypsin and chondroitinase, has been attempted and succeeded in degrading matrix components creating a cavity, inducing the loss of disc integrity (Melrose et al. 1996, Roberts et al. 2008). The injection of cytokines has been also used to stimulate IVD explants to a degenerative environment, by creating an inflammatory setting with significant upregulation of proinflammatory markers (IL-6, IL-8, PGE_2, MMP1, MMP3) and by downregulation of col type II and agg through administration of IL-1, (Teixeira et al. 2016b), or by the injection of TNF-α, that promoted a catabolic shift, agg

degradation and pro-inflammatory cytokines expression (Purmessur et al. 2013, Teixeira et al. 2016b). Strategies relying on mechanical damage, using needle puncture, were shown to induce cell death and alter the disc mechanics, while the partial removal of the nucleus or the annulus can mimic the matrix loss and affect disc integrity (Korecki et al. 2008, Peroglio et al. 2013, Pirvu et al. 2015, Li et al. 2016) Finally, a degenerative state can be achieved by nutritional deprivation and/or unphysiological biomechanical loading that can cause detrimental effects to the IVD, including a significant drop in cell viability (Illien-Junger et al. 2010). These types of *ex vivo* models allow the assessment of several parameters, such as, the production of growth factors, cytokines and metabolic/biosynthetic activity in culture. They can further address the success of cell therapy (direct cell injection, cell-delivery systems using biomaterials) in a more physiological environment, than *in vitro* models. Additionally, *ex vivo* models offer the possibility to be cultured under static conditions, static loading or even dynamic loading using well established bioreactors, able to mimic the spine loadings (Gantenbein et al. 2015).

Different models have been used in the study of cell based therapies that differ on their origin, size (with/without CEPs; only NP tissue) and the methods used for inducing degeneration; with particular focus on MSCs survival and differentiation in the degenerative environment and MSCs contribution for regeneration IVD tissue regeneration. These studies are summarized on Table 7.

Overall, cell-based therapies applied to *ex vivo* studies herein reported presented significant beneficial effect of MSCs in IVD regeneration, namely, in what concerns to matrix expression and synthesis. Still, these models are limited in certain aspects, such as, the lack of an immune response and pain assessment studies, that cannot be performed. Recently, *ex vivo* organ cultures using human IVDs have also been established (Gawri et al. 2011, Walter et al. 2014) and although they are considered of high clinical relevance, the access to human IVD material is very limited and the harvesting times are vital for the success of the organ culture. This model was used recently to address the effect of a short peptide in IVD regeneration (Gawri et al. 2013), still, to date, no cell-based therapies have been studied using human organ cultures.

Table 7 | Cell based-therapies*: ex vivo* studies.

IVD Model	Specie	Model	Therapy	Time	Outputs	Reference
NP Explants						
NP explant	**B**	-	hMSCs	**4W**	↑hMSCs viability (4W) ↑Sox-9 ↑Agg ↑Col type II	(Le Maitre et al. 2009)
Enzymatic induced Nucleotomy						
IVD with CEPs	**P**	Digestion with Chymopapain	MSCs, PRP and MSC/PRP combined	**4W**	↑Chondrogenesis ↑Cell proliferation	(Chen et al. 2009b)
IVD with CEPs	**P**	Digestion with Papain	MSCs	**10D**	↓hMSCs viability	(Chan et al. 2013)
IVD with CEPs	**B**	Digestion with papain	hMSCs /autologous NP cells injection in HA-pNIPAM	**16D**	Gene Expression: ↑ACAN ↑COL2A1 ↑VCAN ↑SOX9 ↓MSCs Viability	(Malonzo et al. 2015)
IVD	**B**	Digestion with trypsin	hMSCs hMSCs + Link N	**14D**	↑GAG content ↑Coll type II ↑ hMSCs survival	(Mwale et al. 2014)
Mechancicaly induced Nucleotomy						
IVD with CEPs	**B**	Nucleotomy	hMSCs injection in HA-pNIPAM	**7D**	MSCs differentiation towards a disc-like phenotype	(Peroglio et al. 2013).
IVD with CEPs	**B**	Nucleotomy	hMSCs seeding in the CEP HAPSDF5 Injection in the nucleotomy site	**2D**	↑hMSCs migration towards the lesion	(Pereira et al. 2014)
IVD with CEPs	**B**	Nucleotomy	MSCs seeding in the CEP	**21D**	In the IVD tissue: ↑Col type II ↑Agg	(Pereira et al. 2016)
IVD with CEPs	**B**	Annulotomy	hMSCs	**14D**	↑Col type V	(Pirvu et al. 2015)
Limited Nutrition						
IVD with CEPs	**B**	Nutritional deprivation and/or unphysiological biomechanical loading and anular puncture	hMSCs and IGF-1-transduced hMSCs	**14D**	↑hMSCs migration towards the lesion ↑Proteoglycans (in the IGF-1-transduced hMSCs)	(Illien-Junger et al. 2012)

B – Bovine; P – Porcine; D – Days; W – Weeks;

In vivo studies. In vivo studies have been widely explored in the IVD research field using many different models, from rat to large animals, such as, cows or primates. In current research, the most common models were developed in pigs, sheeps, dogs and rats. These models represent an upgrade comparatively to *in vitro* and *ex vivo* studies, although none can fully represent the biomechanical properties of the human spine, provided by the upright position. In the case of *in vivo* models, as well as it happens to the health *ex vivo* ones, degeneration has to be stimulated, with the exception of the sand rat (Gruber et al. 2014), which develops natural disc degeneration and some dogs presenting spontaneous disc degeneration (Jeffery et al. 2013). In stimulated degenerative models, different approaches have been reported in the literature, being the most common, the puncture of the IVD; the AF incision can be performed without or with nucleotomy, where the NP is partially removed by aspiration or degraded using enzymatic digestion (Sakai and Andersson 2015). To date, there are over thirty studies that have focused in the MSCs regenerative potential using *in vivo* models of IVD degeneration. Some of those studies details and main conclusions are summarized in Table 8.

Overall, a great part of these studies demonstrated the beneficial effect of MSC-based therapy, namely, in what concerns to disc height improvement and the upregulation of the key components of the IVD matrix. It is relevant to stress out that the timing of the treatment appears to be fundamental for the success of a cell based therapy, as demonstrated in the work of Ho et al. where the severity of disc degeneration impaired the beneficial effects of transplanted MSCs (Ho et al. 2008), as well as the number of MSCs transplanted (Serigano et al. 2010). In the study of Vadalà and colleagues, concerns were also raised in the use of transplanted MSCs, by showing an undesired bone formation resultant from MSCs migration out of the NP area (Vadala et al. 2012) while Li et al. demonstrated that when confining MSCs in microspheres, osteophyte formation risk can be reduced (Li et al. 2014a).

Table 8 | Cell based-therapies: *in vivo* studies.

Model	Therapy	Time	Outputs	Reference
Mice				
Biglycan deficient mice (Spontaneous degeneration)	ADCs	12W	↑Signal intensity ↑Cell survival (up to 12W) ↑Aggrecan ↑New expression of Biglycan	(Marfia et al. 2014)
Tail-looping	mMSCs	12W	↑MSC migration towards the lesion	(Sakai et al. 2015)
Rat				
Disc puncture	*hMSCs*	8W	↑Cell survival ↑Disc Height	(Jeong et al. 2009)
Disc puncture (Caudal discs)	*rMSCs*	2 W	↑Disc Height ↓GLUT-1 expression ↓Herniation	(Cunha et al. 2016)
Disc puncture (Caudal discs)	*MSCs (Overexpressing BMP-7)*	8 W	↑Disc Height in early MSCs injection time point (day 0) ↓Histological score	(Liao 2016)
Disc puncture (Caudal discs)	*rMSCs* *Llocal injection* At day 3, 14 & 30	2 W	↑GAG content in early MSCs injection time point (day 3)	(Maidhof et al. 2017)
Rabbit				
NP Aspiration	*rbMSCs*	8W	↑Cell viability ↑Histology improvement ↑ECM synsthesis	(Sakai et al. 2003)
-	*rMSCs (15% Hyaluronan Gel)*	4W	↑Disc Height ↓MSCs numbers ↑Cell survival	(Crevensten et al. 2004)
-	*rbMSCs*	24W	↑Col type II ↑Proteoglycans	(Zhang et al. 2005)
NP Aspiration	*rbMSCs* *Local Injection in atelocollagen*	48W	↑Cell survival (up to 2W) ↑proteoglycan content ↑matrix-related genes	(Sakai et al. 2005)
NP Aspiration	*rbMSCs* *Local Injection in atelocollagen*	24W	↑Disc Height ↑MRI intensity ↑Protoeglycans	(Sakai et al. 2006)
-	*rbMSCs*	24W	↑MSCs survival ↑Engrafment	(Sobajima et al. 2008)
Disc Puncture	*rbMSCs*	16W	↑Disc Height ↑Proteoglycans	(Ho et al. 2008)
Axial loading	*rbMSCs*	24W	↑IVD regeneration	(Hee et al. 2010)
NP Aspiration	*rbMSCs*	24W	↑ECM synthesis ↓Degradative enzymes ↓Inflammatory cytokines	(Miyamoto et al. 2010)

Table 8 | Cell based-therapies: *in vivo* studies. (Continuation)

NP ASPIRATION	*RBMSCS IN FIBRIN TGF-ß1*	12W	↓APOPTOSIS ↑DISC HEIGHT ↑COL TYPE II	(Yang et al. 2010)
-	*RBMSCS*	16W	↑MRI INTENSITY ↑GAG CONTENT	(Feng et al. 2011)
PUNCTURE	*RBMSCS*	9W	↑MSCS MIGRATION ↑OSTEOPHYTE FORMATION	(Vadala et al. 2012)
PUNTURE	*RBMSCS IN COLLAGEN MICROSPHERES*	24W	↓ OSTEOPHYTE FORMATION	(Li et al. 2014a)
DISC PUNCTURE	*HMSCS*	8W	↑MSCS SURVIVAL	(Tao et al. 2016)
DOGS				
NP ASPIRATION	*DMSCS*	12W	↑MSCS VIABILITY ↑DISC HEIGHT ↑MRI INTENSITY ↑ECM SYNTHESIS	(Hiyama et al. 2008)
NP ASPIRATION	*DMSCS*	12W	↑ECM SYNTHESIS ↓THE NUMBER OF TRANSPLANTED MSCS CAN AFFECT THE OUTCOME	(Serigano et al. 2010)
PIG				
NP ASPIRATION	*PMSCS LOCAL INJECTION HYALURONAN-ENHANCED ALBUMIN HYDROGEL*	3D	↑RETAINMENT AND ↑METABOLICAL ACTIVITY	(Omlor et al. 2014)
NP ASPIRATION	*HMSCS*	24W	↑MSCS SURVIVAL ↑MSCS EXPRESSION OF SOX9 ↑ MSCS EXPRESSION OF COL TYPE II	(Henriksson et al. 2009b)
SHEEP				
POSTERO-LATERAL ANNULOTOMY	*SMSCS*	12 M	↑DISC HEIGHT ↓PFIRRMANN GRADE ↓HISTOPATHOLOGICAL GRADE ↑SPONTANEOUS REPAIR OF THE ANNULAR LESION	(Freeman et al. 2016)

Clinical trials. As a promising therapy for IVD degeneration treatment, the use of MSCs have move forward from the lab bench to *in vivo studies* and further on to the first studies in human patients. The first study, reporting the injection of MSCs in human patients was performed by Yoshikawa et al., as a case report study, in 2010. The regenerative effects of autologous MSCs in markedly degenerated discs, were assessed in two patients. Results showed an improvement of the symptoms 2 years after surgery, while X-ray, Rontgen

kymography and CT showed decreased instability and T2-weighted magnetic resonance indicated high moisture contents (Yoshikawa et al. 2010). Later, in 2011, Orozco et al. conducted a similar study with nine patients. In his study, MSCs transplantation by direct injection of around $10\pm5x10^6$ in the NP, was shown to be feasible, safe and to induce an analgesic effect, by an improvement in pain and in disability, and a further improvement of water content, but no restoration of disc height. Overall, the analgesic effects were faster than the regenerative ones, which might be related to the MSCs well-known immunomodulatory effect. Although promising, this study only presented observations within 1 year after treatment remaining to be evaluated long term effects of the treatment (Orozco et al. 2011). In 2015, two different studies were released, reporting IVD degeneration treatment with cell-based therapies, one using unexpanded autologous MSCs and a second one using reactivated NP cells. In Pettine et al. study, similarly to what was described to Orozco et al, most of the patients reported a significant pain relief within 14 days following BM-MSCs concentrate injection in the NP of symptomatic discs, and this relief was beyond the 12 months of the study. A higher disc hydration was detected by MRI in 8/20 patients, consistent with pain relief. These improvements were more pronounced in patients injected with higher CFU-F (colony forming-unit-fibroblasts) concentration, suggesting a critical dose concentration of fresh, non-cultured MSCs, for IVD treatment of about 2000 cells/mL (Pettine et al. 2015). Mochida et al. presented a 3-year old prospective clinical study, using MSCs-activated NP cells transplantation, in the treatment of lumbar degenerated discs. NP cells were harvested from fused discs, and after activation with autologous MSCs (in co-culture) were injected in fused adjacent disc. In this study, no adverse effects were reported within the 3-years-follow-up and although no detrimental effects of cell transplantation were observed, the treatment revealed a poor efficacy in the improvement of IVD degeneration (Mochida et al. 2015). Very recently, Elabd et al. reported a study involving five patients which undergo intradiscal injection of autologous, hypoxic cultured BM-MSCs. During the 4-6 years' post treatment follow-up, no adverse events were reported and all patients self-reported an overall improvement. Although promising, in terms of safety, this study was performed in a small sample group and further studies are needed to address the clinical relevance of hypoxic cultured BM-MSCs treatment (Elabd et al. 2016). At the moment, there are more than 10 clinical trials registered in the US NIH ClinicalTrials.gov data base, focusing on the applicability of MSCs for intervertebral disc treatment, reflecting the great investment in MSCs-based therapies. Taken together, the data on MSCs beneficial effects both in animal models and humans is promising and safe, although several questions still remain in what concerns to the timing of treatment and long term effects, questions that the ongoing trials might be able to answer in the near future.

CHAPTER II

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CELL RECRUITMENT FOR DEGENERATED INTERVERTEBRAL DISC

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Cell recruitment for degenerated intervertebral disc
to be published *in* Gene and Cell Delivery for Intervertebral Disc Book

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CELL RECRUITMENT FOR ENDOGENOUS TISSUE REPAIR/REGENERATION

Although cell-based therapies represent a promise in the treatment of several disorders, they still face some ethical concerns and risks. Cell transplantation is still associated with immune rejection in the case of allogeneic cell sources (frequently the most accessible and adequate), and the use of autologous cells may lead to further tissue morbidity. Other risks concern the cell characteristics, such as differentiation status, tumorigenic potential and diseases/virus transmission (Herberts et al. 2011). Alternatively, a new trend in the field of regenerative medicine appears, based on the mimicry of healing processes that naturally occur in our body. Stem/progenitor cells can be guided by intrinsic cues from their niche to a lesion site. Nevertheless, endogenous regeneration can be insufficient to achieve a successful tissue repair, but if enhanced, by maximizing the body's own regenerative capacity, it could potentially constitute an alternative strategy to achieve full tissue repair/regeneration.

STEM CELL HOMING AND MIGRATION

Stem cell homing and migration represent similar concepts on stem cells capacity to travel under guided navigation in our body. The homing process, designated following the original word “home”, which commonly stands for residence, is used to designate the natural process by which hematopoietic cells in circulation migrate through the blood vessel and cross the vascular endothelium to the bone marrow (BM) (Lapidot et al. 2005). Depending on the authors and research field, the term homing might also be applied to the capacity of other stem cells to travel to organs in response to stress or injury, as it often happens in the regenerative medicine research field (Naderi-Meshkin et al. 2015). Still, stem cell researchers consider this as the migration/mobilization of these cells towards other tissues and sites of lesion following a stimulus, and not a homing process. This leads to the definition of home: in what concerns the progenitor cells' home/residence, it has been defined in the literature as their niche.

The niche is characterized by a specific microenvironment, with appropriate anatomy and dimensions, that allows stem cells to reproduce and self-renew (Scadden 2006). The BM is the most well-known niche of stem cells, harbouring both haematopoietic stem cells (HSCs), that give origin to all types of blood cells, and non-haematopoietic stem cells, such as mesenchymal stem cells (MSCs), representing less than 0.01% of the BM mononuclear cells, or endothelial progenitor cells (EPCs) (Pittenger et al. 1999).

The BM is described to be the primary site of adult haematopoiesis (Kiel and Morrison 2008). MSCs, residing in the stromal component, have a key role in the support and maintenance of the HSCs stemness, and although they can be found mostly in the BM, other niches have been described (da Silva Meirelles et al. 2006). MSCs can reside in multiple

organs, such the adipose, skeletal and muscle tissue, and participate in the endogenous repair process through their capacity to migrate to injury sites (Liu et al. 2009). MSCs are recognized as key players in this process since they are able to proliferate, differentiate and functionally contribute to the regenerative process (Granero-Molto et al. 2008). Their mobilization to different tissues occurs following a chemotactic stimulus initiated by damage, such as trauma, fracture, inflammation, necrosis or tumour presence (Fong et al. 2011) (Figure 1).

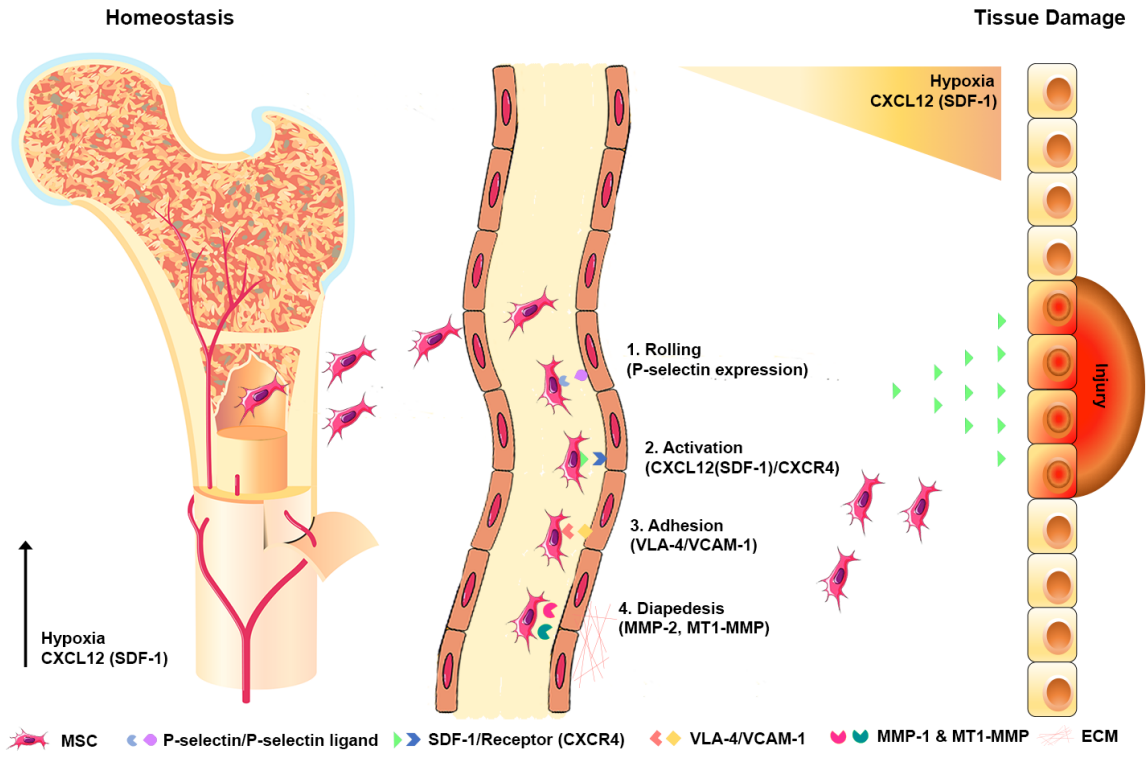

Figure 1 | MSC Mobilization to sites of damage. Mechanisms of MSC transendothelial migration towards injured tissue. MSCs can cross through a mechanism encompassing several phases of (1) rolling (involvement of p-selectin); (2) activation of chemokine receptors; (3) adhesion through adhesion molecules and their ligands ((VLA)-4; endothelial cells express the corresponding ligand, vascular cell adhesion molecule (VCAM)-1). Moreover, MSCs express the extracellular matrix-degrading enzymes, matrix metalloproteinase-(MMP-) 2 and membrane type (MT)1-MMP that play a role in their extravasation and afterwards migration towards the lesion following the chemokine gradient. (Image created for this thesis. Bone section credits: Wikimedia Creative Commons, Pbroks13; MSCs adapted from Andrew Owen, Philip N. Newsome publication on the American Journal of Physiology - Gastrointestinal and Liver Physiology. DOI: 10.1152/ajpgi.00036.2015)

The cell migration process is orchestrated by particular molecules, such as cytokines and chemokines, and involves the participation of several adhesion molecules such as selectins, chemokine receptors and integrins (Butcher 1991, Springer 1994). Generally it is assumed that MSCs mobilization into the peripheral blood follows the same steps that were described for leukocytes and HSCs recruitment to inflammation sites (Fox et al. 2007b), although differences between the recruitment of MSCs and leucocytes have been described. These differences concern L- and E-selectin (Muller et al. 1993), which are low or absent in MSCs and the absence of platelet/endothelial cell adhesion molecule 1 (PECAM-1)/CD31 (Ruster et al. 2006). MSCs bind to endothelial cells in a P-selectin-dependent manner (rolling) (Figure 1) and upon activation, cell adhesion is mediated by the very-late antigen-4 (VLA-4)/VCAM-1 axis, while the extravasation to the matrix is intermediated by matrix degrading enzymes such as MMP-2 and membrane type 1 MMP (MT1-MMP) and tissue inhibitor of metalloproteinase 1 (TIMP-1) (Ries et al. 2007, Karp and Leng Teo 2009).

STEM CELL MIGRATION: CHEMOKINES, RECEPTORS AND GROWTH FACTORS

Cell recruitment is triggered by specific molecules, such as chemokines, cytokines and growth factors, present in a chemotactic gradient (Rankin 2012). The chemokines are small peptides (8-14 kDa) grouped based on the arrangement of the terminal cysteine residues (C) that form the first disulphide bond (Zlotnik and Yoshie 2012). When adjacent, the residues are referred to as CC; when separated by an amino acid (X), they are referred to CXC. Chemokines can be further categorized based on their function: while some chemokines are involved in the leucocyte migration during a pathological condition, others are constitutively expressed in homeostatic conditions to coordinate the location and mobilization of cells (Anders et al. 2014). Until now, more than 40 chemokines have been described (Nomiyama et al. 2013). Chemokine signalling occurs through binding to chemokine receptors (CCR) or to atypical chemokine receptors, which are receptors that lack a specific conserved motif and do not mediate conventional signalling and directional migration (Bachelerie et al. 2014).

CXCL12, first identified as stromal cell derived factor-1α (SDF-1α) in the supernatant of BM stromal cells, is one of the most studied and important chemokine, due to its role in haematopoietic stem and progenitor cell homing and localization in the bone marrow (Rankin 2012). Previous studies reported that SDF-1α activation occurs through two main receptors: CXCR4 and CXCR7. While the SDF-1α/CXCR4 plays an important role in stem/progenitor cell migration, CXCR7 functions as a specific scavenger for SDF-1α, and was described to modulate the activity of the ubiquitously expressed CXCR4, suggesting a key role in the fine-tuning of stem cell mobility (Naumann et al. 2010).

SDF-1α is a potent chemoattractant for HSCs (Aiuti et al. 1997). The importance of the SDF-1α/CXCR4 axis in HSCs homing, maintenance of quiescence and cell mobilization to the blood has been widely documented (Peled et al. 1999). Moreover, HSCs mobilization is intimately associated with SDF-1α levels in the BM. Mice with CXCR4-/- chimeric BM were shown to have high levels of HSCs in the blood (Christopher et al. 2009) and the peaks of SDF-1α in the BM, which fluctuate according to the circadian variation, correlate with higher levels of HSCs in the BM and lower levels in the blood (Mendez-Ferrer et al. 2008). Other studies suggested SDF-1α as a key regulator of HSCs quiescent state under homeostasis. Sugiyama et al. reported a reduction of HSC numbers and increased sensitivity to myelotoxic injury occurring following CXCR4 deletion in the adult mice (Sugiyama et al. 2006). Tzeng et al. showed that the loss of stromal secreted SDF-1α, in a murine with a conditional deletion of SDF-1α in adult stages, led to a reduction of quiescent HSCs (Tzeng et al. 2011). Both studies demonstrated the importance of SDF-1α/CXCR4 signalling pathways in maintaining the quiescent HSC pool. Granulocyte colony-stimulating factor (G-CSF) has also been described to be involved in HSC mobilisation from the BM through the disruption of the SDF-1α/CXCR4 retention axis (Pitchford et al. 2009).

The SDF-1α receptor has also been described to be expressed in EPCs and it is known that its antagonist AMD3100 mobilizes these cells from the BM to the blood (Capoccia et al. 2006, Shepherd et al. 2006). The mobilization of these cells may also occur via the VEGF/VEGFR2 axis, by triggering cell migration (Pitchford et al. 2009) or even through CXCR2, as demonstrated in the work of Jones et al. where this receptor was demonstrated to be critical for EPC recruitment and angiogenic response (Jones et al. 2009).

Mechanisms of MSC mobilization and the chemokines/receptors involved in the process have widely been studied, due to the interest in these cells for tissue regeneration and repair. Like other cells, MSCs express many receptors and cell adhesion molecules that support cell homing and migration to target tissues in response to injury. Through the years, it became generally accepted that the mobilization process of MSCs was highly dependent on the SDF-1α/CXCR4 axis (Figure 1). BM-derived MSCs were shown to express CXCR4 and migrate toward SDF-1α gradients *in vitro* (Ponte et al. 2007). Wynn et al. have shown SDF-1α regulates the migration of MSCs in a dose-dependent manner with maximal migration occurring at 30 ng/mL of SDF-1α (Wynn et al. 2004). Cell migration was also pronounced in cells highly expressing the surface receptor CXCR4 (Wynn et al. 2004). The authors believe that mobilizing the internalized receptor and increasing its functional expression may play a key role in the mechanism of MSC engraftment to bone marrow. Moreover, when inhibiting the receptor CXCR4, MSC migration was shown to be reduced by 46% (Wynn et al. 2004). Similar findings were found *in vivo*, where SDF-1α/CXCR4 axis played a crucial role in BM-MSC

migration to a fracture site. SDF-1α expression was increased during the healing process of live bone suggesting it to be a key regulator in bone repair. Adding to this, the treatment of a fracture model of mice with SDF-1α improved cell migration towards the lesion, which was inhibited in the presence of a CXCR4 antagonist (Kitaori et al. 2009). Other studies further reported an increase in SDF-1α expression following facture both in rats and in plasma of human patients 2-4 days after the lesion (Kidd et al. 2010, Lee et al. 2010b), adding further evidence that the SDF-1α/CXCR4 pathway may have an important role in fracture healing (Yellowley 2013). Indirectly, MSC migration might also be triggered by hypoxia and expression of the hypoxia-induced factor-1 (HIF-1α) in damage sites, which by their turn may drive an upregulation of SDF-1α in the tissue (Ceradini et al. 2004) (Figure 1).

In spite of being, by far, the most well studied receptor of SDF-1α, the expression of CXCR4 in progenitor cells from BM is still controversial. Several studies demonstrated that CXCR4 expression is predominantly intracellular, while others defend the opposite (Honczarenko et al. 2006, Pelekanos et al. 2014). Pelekanos et al. compared the CXCR4 expression of both foetal bone marrow derived MSCs and adult cells. A very low expression ($3.8 \pm 0.3\%$) of the receptor was detected by immune staining at the cell surface of foetal MSCs, however, when permeabilized, 50 to 90% of the cells stained positive for intracellular localization of CXCR4; this pattern was also found in adult MSCs, suggesting that this phenomenon is independent of cell maturity (Pelekanos et al. 2014). On the other hand, the work of Honczarenko and colleagues reported a surface expression of around 43% of the CXCR4 receptor in adult MSCs at early passages (passage 2), as assessed by flow cytometry and further confirmed by RT-PCR (Reverse transcription polymerase chain reaction). However, at later passages (12-16 passages) there was a decrease in the cell surface receptors that resulted in the lack of responsiveness to chemokines. This loss was further accompanied by the decrease of several other molecules known to be involved in the migration process, such as adhesion molecules (ICAM and VCAM) (Honczarenko et al. 2006). CXCR4 expression is dynamically regulated by external cues like hypoxia (Schioppa et al. 2003), and can be up-regulated in adult MSCs following *in vitro* priming with a mixture of cytokines (Shi et al. 2007) or via viral transduction. Other cell surface molecules, such integrin-β1, integrin-α4 and integrin ligands VCAM and ICAM, were found to be expressed by MSCs and also have a key role in the migration process and in the interaction with endothelial cells (Ruster et al. 2006, Ip et al. 2007).

Although most of the current literature concerns the importance of SDF-1α and its receptor CXCR4, MSCs have been shown to express a wide number of chemokine receptors on the cell surface, which also play a role in the migration and guidance of the cells (Honczarenko et al. 2006), such as CXCR1, CXCR2, CXCR3, CXCR5, CXCR6, CX3CR1,

CCR1, CCR3, CCR5, CCR7, CCR9 and CCR10 (Fox et al. 2007a); though, the heterogeneity of MSC populations does not allow the establishment of a specific repertoire of receptors and expression levels in culture, since they may vary with cell isolation, culture conditions and passages (Honczarenko et al. 2006). Moreover, MSCs expansion might lead to changes in MSCs phenotype and to different chemokine receptors repertoire (Bara et al. 2014).

Besides the aforementioned cytokines, other molecules are also involved in MSCs recruitment to injured tissues. Growth factors, such as platelet-derived growth factor (PDGF)-BB, PDGF-AB, epidermal growth factor (EGF), Heparin-binding epidermal growth factor-like growth factor (HB-EGF), TGF-β, IGF-I, hepatocyte growth factor (HGF), bFGF and thrombin have been reported to enhance MSC migration in appropriate concentrations (Li et al. 2007, Ozaki et al. 2007, Ponte et al. 2007). Monocyte chemotactic protein-1 (MCP-1) was also described to have a role in stimulating MSC migration in response to a primary breast tumour in mice (Dwyer et al. 2007), while MCP-3 was suggested as a potential recruitment factor for MSCs towards the heart (Schenk et al. 2007).

IMMUNE CELL MIGRATION: CHEMOKINES, RECEPTORS AND GROWTH FACTORS

Similar to what has been described in the mobilization process of stem cells, chemokines and their receptors play an important role in immune cell mobilization and localized response (Surmi and Hasty 2010). These factors do not only guide the cells to target sites of infection and inflammation; they also coordinate the interaction between these cells, thereby providing the appropriate and optimal adaptive immune response against pathogens, tumour cells or dead cells (Sokol and Luster 2015). For example, in immune cell development, SDF-1α/CXCR4 interactions remain essential for BM retention and normal development of several immune cells, such as B cells, monocytes, macrophages, neutrophils, natural killer cells and plasmocytoid dendritic cells (Mercier et al. 2011). Monocyte exit from the BM seems to be dependent on CXCR4 and CCR2 in homeostatic conditions. Monocytes may further differentiate into proinflammatory (CCR2+) or anti-inflammatory (CX3CR1+) subsets. CX3CR1 expressing monocytes are thought to migrate to the periphery under CX3CL1 gradients and might develop into tissue macrophages following additional chemokine signals (Sokol and Luster 2015). Eosinophils released from the bone marrow to the peripheral tissues are largely dependent on CCL11/CCR3 interactions, while basophils release are mainly mediated by CXCR4, although they constitutively express CXCR1, CCR1, CCR2 and CCR3 (Palframan et al. 1998, Iikura et al. 2001).

STRATEGIES TO ENHANCE CELL MIGRATION

Extending the body's inherent repair capacity by enhancing the mobilization of endogenous cells to participate in the regenerative process has been an attractive therapeutic strategy explored in diverse tissues. This goal can be achieved either by: increasing the local concentration of a certain chemoattractant, using delivery systems or direct protein injection; or by overexpression of chemokine receptors in transplanted cells, to better respond to the recruitment stimuli of the lesion. Both strategies will be further discussed in the next sections.

Increasing the local concentration of chemokines in a certain tissue can be challenging due to the chemokines short half-life and rapid diffusion that can impair the formation of a gradient capable of promoting cell chemotaxis. Following these limitations, the development of delivery systems capable of protecting peptides from being cleaved by proteases and providing a sustained release became indispensable. In the past years, several chemokine delivery systems were developed for a variety of applications, such as guided immunotherapy and tissue regeneration.

CHEMOKINE DELIVERY SYSTEMS TO PROMOTE STEM CELL RECRUITMENT

The great majority of the studies using chemokine delivery systems were developed for guided tissue regeneration and were focused on the SDF-1α/CXCR4 axis. These studies include *in vitro* and *in vivo* delivery of SDF-1α using different delivery systems/biomaterials (PLGA, alginate, collagen, etc) for the recruitment of transplanted or endogenous cells towards damage tissues, such as bone, cartilage, tendons, heart and wound healing. *In vitro* the ability of SDF-1α to be released from delivery systems and recruit MSCs has been widely demonstrated (Thieme et al. 2009, He et al. 2010, Cross and Wang 2011, Goncalves et al. 2012). *In vivo*, numerous studies have applied the delivery of SDF-1α for tissue regeneration, some of those studies are summarized on table 1.

Besides promoting cell recruitment towards the damaged tissue, in the majority of the cases, a positive effect on the tissue was reported (Table 1), reinforcing the role and the participation of this chemokine in tissue repair and introducing new perspectives on the stimuli of endogenous repair capacity. To date, there are no published or ongoing clinical trials using SDF-1α delivery platforms; still, there are two clinical trials, one completed and one recruiting, which uses JVS-100, a non-viral gene that expresses SDF-1α for the treatment of peripheral arterial disease and critical limb ischemia (ClinicalTrials.gov Identifier: NCT02544204 and NCT01410331, respectively).

Table 1 | *In vivo* studies using SDF-1 delivery systems for tissue regeneration.

Material	Chemokine	Mode	Cells	Outcomes	Reference
Bone					
PLC/gelatine membrane	SDF-1α	Rat Cranial Defect	**rBM-MSCs GFP-rBM-MSCs**	↑Bone formation	(Ji et al. 2013)
Collagen Membrane Scaffold	SDF-1α	Periodontal bone defect in the rat mandibular bone	**Host cells**	↑MSC/HSC cell engraftment ↓Inflammatory response ↑Osteoclastogenesis, angiogenesis ↑Bone regeneration.	(Liu et al. 2015)
Cartilage					
Collagen type I sponge scaffold	SDF-1α	Partial- and full-thickness defect in Rabbit	**C-hMSCs SM-hMSCs**	↑Migration and adhesion of C/SM-MSCs	(Zhang et al. 2013a)
Ultrapurified alginate gel (UPAL gel)	SDF-1α	Full-thickness osteochondral defect in rabbit	**Host cells**	↑Migration of host BM MSCs ↑ Histological scores ↑Compressive modulus of reparative tissues	(Sukegawa et al. 2012)
HA-PNIPAM hydrogel	SDF-1α	IVD nucleotomy	**hMSCs**	↑Migration	(Pereira et al. 2014)
Tendon					
Silk-collagen sponge scaffold	SDF-1α	Achilles tendon injury model	**Host cells**	↑Neotendon tissue repair ↑CXCR4-expressing cells ↓Inflammatory cells.	(Shen et al. 2010)
Skin					
Alginate scaffolds	SDF-1α	Porcine wound healing model	**Host cells**	↑Wound healing ↓Scar formation	(Rabbany et al. 2010)
Gelatine hydrogel	SDF-1α + SEW2871	Mouse skin defects (wound healing)	**rBMSCs**	↑MSCs and macrophages recruitment ↑Wound healing	(Kim and Tabata 2016)
Heart					
Hyaluronic acid based hydrogel	SDF-1α (+Ac-SDKP)	Myocardial infarction model in rat	**Host cells**	↑Heart function ↑Angiogenesis ↓Infart size	(Song et al. 2014)
HA-based hydrogel	ESA	Myocardial InfarctionIn rat	**Host cells**	↑Angiogenesis ↓Infart size	(MacArthur et al. 2013)
PEGylated Fibrinogen	SDF-1α	Acute Myocardial InfarctionIn mice	**Host cells**	↑c-kit (+) cells ↑Left ventricular Function	(Zhang et al. 2007)
Hyaluronic Acid Hydrogel	SDF-1α	Myocardial Infarction in mice	**BM-Cells**	↑BM-Cells Homing	(Purcell et al. 2012)

SDF-1 is, by far, the most well studied and applied chemokine to enhance stem cell migration towards a damage site, however, the delivery of other molecules have also been explored.

Lee et al. (Lee et al. 2010a) used a poly-epsilon-caprolactone scaffolf for the delivery of TGF- β3 in the treatment of articular synovial joint in a rabbit model. This system was proven to be efficient in recruiting host cells towards the lesion and contribute for tissue regeneration (Lee et al. 2010a). Basic FGF (bFGF) delivery from collagen hydrogels, alone or in combination with other growth factors such as VEGF, PDGF and nerve growth factor (NGF), has also been applied to dental pulp regeneration, resulting in the re-cellularization and re-vascularization of the damage tissue (Kim et al. 2010)

CHEMOKINE RECEPTOR OVEREXPRESSION

CXCR4, the well-known receptor for SDF-1α/CXCL12 is highly expressed in MSCs when in the BM, decreasing with *in vitro* expansion (after passage two) (Wynn et al. 2004). Thus, the normal laboratory routine in culturing MSCs impairs cell migration towards an SDF-1α gradient. One of the strategies to overcome this is to genetically modify MSCs to increase CXCR4 expression, thus enhancing the migration of systemically delivered MSCs towards the damaged tissue. This strategy has been applied in different studies, with promising results.

MSCs overexpressing CXCR4 via retroviral (Cheng et al. 2008) or adenoviral (Zhang et al. 2008) transduction were shown to enhance *in vivo* mobilization and engraftment of MSCs into an ischemic area where these cells promoted neo-myoangiogenesis and alleviated early signs of left ventricular remodelling (Cheng et al. 2008, Zhang et al. 2008). Thieme et al. demonstrated both *in vivo* and *in vitro* that transient overexpression of CXCR4 in human MSCs induced by transfection, enhanced SDF-1-directed chemotactic capacity to invade internal compartments of porous 3D col type I scaffolds (Thieme et al. 2009). In the study of Ma et al. (Ma et al. 2014), genetically-modified MSCs expressing CXCR4 through lentiviral transduction, migrated to the liver in larger numbers and conferred better functional recovery in damaged liver (Ma et al. 2014).

Although the results are encouraging, genes overexpression engages several risks: gene transfer based on viral vectors is still questionable for clinical applications (Sadelain 2004). An alternative would be the transient exogenous gene transfection, that has the advantage of being rapidly degraded, limiting the duration of overexpression and thus reducing the risk of mutagenic events (Ryser et al. 2008).

IMMUNE CELLS RECRUITMENT

Although to a small extent, the delivery of chemokines aiming to recruit immune cells has also been the focus of some studies, which reported different materials for the delivery of chemokines such as CCL20 and CCL19, aiming to enhance cell migration.

Zhao et al. combined CCL20 and poly(lactide-co-glycolide) (PLGA) microspheres to induce chemotaxis of dendritic cells (DCs). DCs could be attracted towards the microspheres, suggesting that they could be effective for enriching DCs at an immunization site *in vivo* (Zhao et al. 2005). Similar strategy was reported by Singh et al. using crosslinked hydrogels comprising dextran vinyl sulfone and reactive PEG combined with CCL20, for DCs recruitment (Singh et al. 2009). Chemokine sustained release from the gels attracted 4-6 folds more DCs than a single dose (Singh et al. 2009). Alginate microspheres have also been used to load and release several chemokines important in immunity, including CCL19, CCL21, CXCL12 and CXCL10 (Wang and Irvine 2011).

CELL MIGRATION IN THE INTERVERTEBRAL DISC – A NEW PARADIGM?

INTERVERTEBRAL DISC DEGENERATION: CYTOKINES AND CHEMOKINES

The process of IVD degeneration is characterized by the release of several inflammatory cytokines, chemokines and growth factors. Among the secreted proinflammatory mediators are TNF-α, IL-1 α/β, IL-6, IL-17 (Risbud and Shapiro 2014) and chemokines such as CCL5/RANTES (Pattappa et al. 2014), that were reported to play a key role during disc degeneration and may trigger the migration of cells able to modulate inflammation and participate in tissue repair. Both IL-1 and TNF-α are known to induce significant cellular and matrix changes by increasing the expression of matrix degrading enzymes such as MMP-3, MMP-13 and ADAMTS-1, while decreasing the expression of matrix related genes (col type I and II and agg) (Le Maitre et al. 2005, Seguin et al. 2005) in the IVD. On the other hand, the inflammatory cytokines can also stimulate discs to produce chemotactic factors, able to recruit immune cells, such as (MCP)-1, CCL3, CCL4, CCL5, MCP-3, MCP-4, CXCL10, among others.

A summary of cytokines and chemokines identified to date as being expressed during disc degeneration is presented in table 2. Among several factors, CCL5/RANTES has been identified by Pattappa et al. together with CXCL6, in the conditioned media of degenerated IVD organ cultures using proteomic analysis (Pattappa et al. 2014). In migration assays, the CCL5- and CCL5/CXCL6-immunoprecipicitation resulted in a reduced MSC migration, while the CXCL6-immunoprecipicitation did not affect MSC chemotaxis. In addition, mRNA

expression analysis of MSCs cultured in IVD degenerative media, revealed a significant increase in the CCL5 receptors, CCR1 and CCR4 expression. CCL5/RANTES expression in the tissue of both bovine and human discs was further confirmed by immunohistochemistry (Pattappa et al. 2014).

In the study of Kepler et al., the expression levels of CCL5/RANTES, IL-1β, IL-6, and IL-8 in painful discs removed from human patients were analysed by quantitative RT-PCR (Kepler et al. 2013). The authors described that CCL5/RANTES was significantly elevated in painful discs when compared to controls and this increase was correlated with an increased expression of IL-1β (Kepler et al. 2013). These results were further corroborated by a recent study by Grad S et al. which investigated the levels of CCL5 and CXCL6 in the Hong-Kong Disc Degeneration Population-Based Cohort of Southern Chinese (Grad et al. 2016). Levels of both chemokines were elevated in blood plasma samples of subjects with disc degeneration when compared to non-degenerated individuals, associating for the first time CCL5/RANTES and CXCL6 with moderate/severe lumbar disc degeneration and suggesting that these chemokines could be used as systemic biomarkers for the diagnosis and monitoring of disc degeneration (Grad et al. 2016).

CCL3, also known as macrophage inflammatory protein (MIP)-1α, was shown to be upregulated in NP cells isolated from rat and human IVD tissue following treatment with IL-1β or TNF-α. In the same study, CCL3 expression in human samples was shown to be correlated with the grade of tissue degeneration (Wang et al. 2013). This molecule induced macrophages migration after treatment with NP cells conditioned media (previously treated with IL-1β or TNF-α); migration was suggested to occur via CCR1, the primary receptor of CCL3, since its inhibition resulted in cell migration blockage (Wang et al. 2013). CCL2 and CCL3 gene expression were shown to be significantly up-regulated in both human AF and NP cells after treatment with IL-1β (Wang et al. 2013, Liu et al. 2017b). Additionally, some of those chemokines, such as CCL2, CCL7 and CXCL18 have been correlated with histological degenerative tissue changes (Phillips et al. 2013) and are known to be involved in the recruitment of immune cells to inflammatory sites (Luster 1998).

CCL20 expression in NP cells has also been reported. Both CCL20 and its receptor CCR6 have been associated with IVD degenerative conditions (Zhang et al. 2013b). CCR6, the only receptor of CCL20, is specifically expressed on the Th17 cell surface and is related to the recruitment of these cells in several inflammatory diseases; higher levels of IL-17 (T-helper 17, Th17 associated cytokine) have been observed in patients with IVD degeneration (Shamji et al. 2010). This interaction was reinforced in a study using a rat model; Zhang Y. et al, was able to show a positive correlation between the expression levels of CCL20, CCR6 and IL-17-producing cells, suggesting that the recruitment of these cells to degenerated IVD tissues occurred via the CCL20/CCR6 system *in vivo* (Zhang et al. 2016).

An association between AF cell migration and CXCL10 had already been established, suggesting a role of this chemokine in AF homeostasis and repair (Hegewald et al. 2012).

Taken together, these results demonstrate the involvement of several molecules in the disc degeneration pathogenesis. Moreover, most of the studies were able to identify cytokines and chemokines that are directly or indirectly related to the recruitment of immune cells, thereby intensifying the inflammatory response and the release of neurotrophines, promoting pain. The study of these molecules is not only relevant for the design of new therapies, by targeting symptomatic discs and the associated inflammation, but also for understanding the players in stem cell recruitment for repair.

Table 2 | Cytokines and Chemokines identified in the intervertebral disc

Molecule	Specie	Model	Role	Reference
Cytokines				
IL-1	**H**	Degenerated and herniated discs	Chemotactic for neutrophils	(Kepler et al. 2013) (Risbud and Shapiro 2014)
IL-6	**H**	Human Discs	Chemotactic to T-Cells	(Kepler et al. 2013)
IL-8	**H**	Herniated Discs	Chemotactic to neutrophils	(Ahn et al. 2002) (Team et al. 2009)
IL-17	**H**	Human discs	Chemotactic for monocytes & neutrophils	(Shamji et al. 2010)
TNF-α	**H**	Degenerated and herniated discs	Chemotactic neutrophils	(Risbud and Shapiro 2014) (Team et al. 2009)
Chemokines				
CCL2/ MCP-1	**H**	Human Discs	Chemotactic for lymphocytes and mononuclear cell	(Phillips et al. 2013)
CCL3	**H R**	NP Cells	Chemotactic for Immune cells	(Wang et al. 2013)
CCL5/RANTES	**B**	Organ culture IVD with CEPs	Chemotactic for MSCs	(Pattappa et al. 2014)
	H	Human Discs	-	(Kepler et al. 2013)
	H	Blood plasma samples	-	(Grad et al. 2016)
CCL7/ MCP-3	**H**	Herniated lumbar disc specimens	Chemotactic for Immune & Stem Cells	(Phillips et al. 2013) (Kawaguchi et al. 2002a)
CCL20	**H**	Pathological discs	Chemotactic to Th17 cells	(Zhang et al. 2013b)
CXCL9	**H**	Human Discs	-	(Phillips et al. 2013)
CXCL6	**B**	Organ culture IVD with CEPs	Chemotactic for Immune & MSCs	(Pattappa et al. 2014)
	H	Blood plasma samples	-	(Grad et al. 2016)
CXCL10	**H**	AF Cells	Chemotactic for AF cells	(Hegewald et al. 2012)
CXCL12/SDF-1	**B**	Organ culture IVD with CEPs (nucleotomy)	Chemotactic for MSCs	(Pereira et al. 2014)

CELL RECRUITMENT TOWARDS THE IVD

The ability to recruit cells to several tissues by the delivery of chemokines has widely been explored in different tissues; whereas in the IVD, mainly due to its avascular nature, the migration/recruitment of cells is still at the beginning of being explored.

Endogenous Repair. The results published in 2012 by Illien-Junger et al, were a game changer in the IVD regenerative therapy, by demonstrating for the first time that MSCs could be recruited and migrate towards discs cultured under degenerative conditions, throughout the secretion of chemokines by the IVD tissue (Illien-Junger et al. 2012). Later on, Pattappa et al. described CCL5 and CXCL6 as the main responsible chemokines for hMSCs migration to the degenerative discs (Pattappa et al. 2014). Furthermore, Henriksson et al. proposed a migration route from stem cell niches around the IVD towards AF and NP, by providing evidence of gradual migration of proliferating cells (BrdU+), that expressed both cell migration molecules such as SLUG, SNAI1, β1-INTEGRIN and pre-chondrogenic markers as GDF5 and SOX9 (Henriksson et al. 2012). These proliferating cells were first reported as stem cells based on the expression of certain progenitor markers, such as Notch1, Delta4, C-KIT, Stro-1, along with the proliferation marker Ki67. The presence of these cells was confirmed in several mammals (rat, porcine and human tissues), with similar pattern and frequency, suggesting interspecies similarity and demonstrating the presence of progenitor cells within the disc and stem cell niches in the IVD region (Henriksson et al. 2009a). More recently, the same group demonstrated the presence of stem cells (CD90+, OCT3/4+), pre-chondrocytic cells (GDF5+), cells expressing migration markers (SNAI1+, SNAI2+), catabolic markers (MMP9+, MMP13+), inflammatory marker (IL1R+) and adhesion markers (DDR2+, β1-INTEGRIN +) in the previously proposed migration route around the IVD region (Henriksson et al. 2015). Other molecules, such as MMP-9, MMP-12, DDR2 and β1-INTEGRIN, suggest that ECM turnover and cellular motility occurs, demonstrating the role of ECM composition and architecture in stem cell migration (Henriksson et al. 2015).

The isolation of MSC-like cells from the different areas of the IVD (AF, NP and CEP) was recently demonstrated. These cells were compared regarding their proliferation, passage, colony formation, migration, and invasion capacity by the group of Liu et al. (Liu et al. 2017a). Interestingly, the three types of cells presented similar characteristics of proliferation, passage and colony formation capacity; despite cells isolated from the CEP revealed higher migration capacity together with higher expression of CXCR4 (Liu et al. 2017a).

Liu et al. demonstrated the expression of CCR5 at the RNA level in both NP and AF cells (Liu et al. 2017b). Adding to this, in the presence of CCL5/RANTES, increased levels of extracellular signal-regulated kinase (ERK) phosphorylation were detected along with AF cell

migration, suggesting that CCR5 receptor in AF cells is functional and therefore AF cells may have the ability to migrate in response to disc damage or inflammation (Liu et al. 2017b).

Overall, the presence of progenitor cells in different locations of the healthy and degenerated IVD opens new perspectives regarding the possibility for the mobilization/activation of these populations towards IVD repopulation (Risbud et al. 2007, Henriksson et al. 2009a, Sakai et al. 2012). These studies, by demonstrating the existence of closer stem cell niches and by supporting the idea that chemokines and chemokine-receptors might be involved in the migration process to damaged discs, have given new insights on the endogenous repair capacity of this tissue.

Strategies to enhance repair. As endogenous repair has demonstrated to be insufficient in restoring IVD upon damage/degeneration, there was a growing interest in strategies to enhance endogenous IVD repair capacity in the past years.

Pereira et al. investigated the potential of hMSCs seeded on the cartilaginous endplate (CEP), to migrate towards a damaged IVD. These cells were able to migrate and contribute to ECM remodelling by improving the expression of col type II and agg in the disc (Pereira et al. 2016). The same group, have also explored injection of a chemoattractant delivery system to enhance MSC migration towards the IVD, as previously used in different contexts aforementioned (Table 1). Using an *ex vivo* organ culture of nucleotomized IVDs, the sustained delivery of SDF-1 from an HA-based hydrogel was demonstrated. SDF-1 release significantly increased MSCs migration from the CEP, towards both the NP and AF of the disc. This was not the case when SDF-1 was directly administrated to the IVD tissue, suggesting that the delivery system had a key role in protecting the chemokine against degradation and therefore guaranteeing its biological function. Moreover, the number of cells migrating towards the IVD was affected by the MSC donor's age: cells provided by older donors migrated in fewer numbers when compared to cells from younger donors (Pereira et al. 2014).

Wei et al. proposed another approach to improve IVD repair based on the SDF-1/CXCR4 axis, using the transplantation of CXCR4-overexpressing MSCs (Wei et al. 2016). Wei et al. transplanted CXCR4-MSCs into a rabbit punctured disc and was able to track them till 16 weeks' post-transplantation. Overexpression of CXCR4 promoted MSC retention in the IVD and enhanced IVD regeneration (Wei et al. 2016).

Although not addressing directly a strategy to enhance repair, Tam et al. work focussed on the migration of multipotent stem cells upon delivery using two different methods, in a model of mouse caudal puncture-induced degeneration (Tam et al. 2014) and described the advantages of one method over the other, to stimulate IVD repair. Stem cells were injected intravenously or intradiscally and both methods resulted in an increase expression of agg, although only the direct injection contributed to improve IVD morphology and disc height. This

is partially explained by the limited migration to the site of lesion of cells injected intravenously, as only few cells could migrate towards the IVD. Still, a positive effect was observed using both methods, suggesting a paracrine effect of the stem cells, and though few cell have reached the disc, this indicate that *in vivo* the lesion itself might trigger cell recruitment (Tam et al. 2014).

In 2015, Sakai et al. published a pilot study which aimed to assess *in vivo* the mobilization of MSCs to the disc (Sakai et al. 2015). In this study, a BM-chimeric mouse expressing enhanced green fluorescent protein (GFP) was used and disc degeneration was induced by tail-looping, thus establishing a new model of IVD degeneration. The results provided first evidence of MSCs dynamic response following disc degeneration but also reinforced the importance of vasculature in this process, by showing that BM-MSC distribution decreased with increased distance from the vascularized areas (growth plate and CEP). In the nucleus aspiration group, the needle punch seemed to favour cell migration into the disc via the AF; still, when no AF rupture was present, the migration was more likely to occur through the CEP, which can itself also represent a barrier in limiting cellular penetration into the IVD (Sakai et al. 2015). The avascular nature of the IVD was, again, shown to be one of the biggest limitations of this tissue's self-repair and regenerative potential.

Although few studies have addressed strategies to enhance IVD repair, so far, the results obtained are encouraging and will certainly contribute for the development of new approaches for IVD repair stimulation using chemokines or growth factors that may enhance or accelerate endogenous cell migration towards the disc.

CHAPTER III

CHAPTER III

AIM AND OBJECTIVES

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The aim of this thesis was to contribute for an alternative strategy for Intervertebral Disc (IVD) regeneration through stem cell recruitment. To achieve this purpose, an organotypic *ex vivo* culture of the IVD, containing the cartilaginous endplates (CEP) was used as model to test an alternative route for MSCs based therapies in the treatment of IVD degeneration and to explore the feasibility of a chemoattractant delivery system to enhance the recruitment of MSCs to the IVD. For that, the work was divided in three main objectives:

1. To investigate the ability of MSCs to migrate from the CEP into the damaged IVD organ culture and their role in the matrix remodelling of IVD;

2. To address the feasibility of improving MSCs recruitment from the CEP to the IVD using a chemoattractant delivery system based on HA and SDF-1;

3. To evaluate the effect of MSCs recruitment enhancement by SDF-1 in accelerating and/or improving the ECM remodelling in IVD.

CHAPTER IV

CHAPTER IV - ARTICLE 1

MESENCHYMAL STEM/STROMAL CELLS SEEDED ON CARTILAGINOUS ENDPLATES PROMOTE INTERVERTEBRAL DISC REGENERATION THROUGH EXTRACELLULAR MATRIX REMODELING

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Mesenchymal Stem/Stromal Cells seeded on cartilaginous endplates promote Intervertebral Disc Regeneration through Extracellular Matrix Remodeling

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ABSTRACT

Intervertebral disc (IVD) degeneration is characterized by significant biochemical and histomorphological alterations, such as loss of extracellular matrix (ECM) integrity, by abnormal synthesis of ECM main components, resultant from altered anabolic/catabolic cell activities and cell death. Mesenchymal Stem/Stromal Cell (MSC) migration towards degenerated IVD may represent a viable strategy to promote tissue repair/regeneration. Here, human MSCs (hMSCs) were seeded on top of cartilaginous endplates (CEP) of nucleotomized IVDs of bovine origin and cultured *ex vivo* up to 3 weeks. hMSCs migrated from CEP towards the lesion area and significantly increased expression of col type II and agg in IVD, namely in the nucleus pulposus. Concomitantly, hMSCs stimulated the production of growth factors, promoters of ECM synthesis, such as fibroblast growth factor 6 (FGF-6) and 7 (FGF-7), platelet-derived growth factor receptor (PDGF-R), granulocyte-macrophage colony-stimulating factor (GM-CSF) and insulin-like growth factor 1 receptor (IGF-1sR). Overall, our results demonstrate that CEP can be an alternative route to MSC-based therapies for IVD regeneration through ECM remodeling, thus opening new perspectives on endogenous repair capacity through MSC recruitment.

INTRODUCTION

The unique and complex structure of the intervertebral disc (IVD) confers to it exclusive features, such as the capacity to support the whole body weight and a wide range of movements/loadings on the spine. The IVD is an avascular organ composed of a gel-like central part, the nucleus pulposus (NP), surrounded by a lamellar fibrous structure, the annulus fibrosus (AF), and the cartilaginous endplates (CEP), which link each disc to the adjacent vertebral bodies[1]. With ageing, IVD undergoes a degeneration process, in which mismatch between anabolic and catabolic processes orchestrate an alteration of matrix composition, which differs from extracellular matrix (ECM) of healthy IVD[2]. During IVD degeneration several histomorphological changes occurs, including NP fibrosis, loss of lamellar organization of the AF, and increased cell death and senescence. In addition, the ability of the IVD to support mechanical forces and to provide flexibility and mechanical stability to the spine becomes compromised due to a loss ECM integrity, caused by abnormal synthesis of its main components–collagen, proteoglycans–and a great loss of water content. Altogether, these alterations culminate in the loss of IVD biological function[3].

Current treatments for IVD degeneration focus on painful degenerative discs and involve conservative approaches or in more severe situations, surgical procedures such as spine fusion or IVD replacement. However, these strategies can affect spine biomechanics and are not able to restore the IVD biological function; besides triggering degeneration of adjacent discs[4]. Alternative strategies based on biomechanically-competent hydrogels[5] that share similar composition to disc ECM have been attempted, but failed mostly due to material extrusion from AF after implantation[6]. From another perspective, cell-based therapies for degenerated IVD have quickly grown over the past years, namely using Mesenchymal Stem/Stromal Cells (MSCs)[7]. MSCs were shown to differentiate into NP-like cells[8,9]. *In vitro*, MSC co-cultured with NP cells associate with both up-regulation of SOX-9 and ECM proteins, in particular aggrecan (Agg) and collagen type II (Col type II), dependent on direct cell-cell contact[8,10,11]. *In vivo*, MSCs were already transplanted to IVD in different animal models, as extensively reviewed by Sakai and Andersson[12]. The promising results obtained from those studies, such as the higher expression of Col type II and disc height recovery[12], have encouraged clinical trials based on MSC transplantation as treatment for degenerated IVD and low back pain[13,14]. The published clinical trials have shown significant pain reduction, a partly recovery of disc hydration, and few signs of IVD regeneration[15]. In fact, cell transplantation through the AF faces several problems: first, the injection itself can trigger IVD degeneration[16]; second, the high IVD pressure can lead to cell leakage and trigger osteophyte formation[17]; and third, the hostile environment of degenerated IVD can induce cell death post-injection[18].

Therefore, the CEP appears as an alternative route to deliver therapeutic agents to the degenerated IVD. By using a new surgical method, the transpedicular approach, Vadalá *et al*.[19,20] showed that the NP can be approached through the CEP via pedicle without affecting the spinal canal and the neural foramina. This route can be an alternative path to reach the NP, without AF disruption, which is one of the major problems of IVD surgical approaches.

In the past few years, several arguments were raised stressing the role of the CEP in accessing the NP to treat IVD degeneration. Cells with stem cell-like characteristics were described in the CEP of degenerated human IVDs[21]. CEP stem cells were shown to differentiate into osteogenic and chondrogenic lineages, in a rabbit IVD degeneration model, suggesting their applicability in NP tissue engineering[22,23]. Moreover, Henriksson, *et al*.[24,25] described for the first time the presence of slow cycling cells and a stem cell niche around adult IVD region and suggested that these cells can migrate towards the AF and inner parts of the IVD. On the other hand, Illien-Jünger and colleagues[26] demonstrated that *ex vivo* cultures of IVDs in degenerative conditions secrete chemokines that specifically recruit MSCs and not fibroblasts. Furthermore, our own group showed that incorporation of the chemoattractant Stromal Cell Derived Factor-1 (SDF-1) into a hyaluronic acid hydrogel, promotes MSC migration from the CEP to the NP and AF[27].

In this study, we investigated the role of human MSCs (hMSCs) seeded on CEP in IVD tissue remodeling, using long-term *ex vivo* cultures of nucleotomized IVDs. We hypothesized that repopulation of the IVD with healthy cells has the potential to restore tissue homeostasis and reverse the degenerative process. Although an enormous challenge, a strategy that could stop/revert IVD degeneration, without damaging the AF, would be of great relevance.

MATERIALS & METHODS

Human Mesenchymal Stem Cells Culture

hMSCs were obtained from discarded human BM tissues from patients undergoing total hip arthroplasty. Patients gave informed written consent for tissue use for research purposes and procedures were carried out in accordance with the relevant guidelines approved by the Centro Hospitalar São João Ethics Committee. All samples were analyzed with patient data coded. hMSCs were obtained from two different donors with ages of 45 and 49 years and isolated by density gradient centrifugation and adherence to tissue culture plastic as previously described70. MSC isolation was confirmed by flow cytometry (cells were CD105, CD73 and CD90 positive, while CD45, CD34, CD14, CD19 and HLA-DR negative), and capacity to differentiate into osteoblasts, chondroblasts or adipocytes70 (data not shown). hMSCs were expanded in low-glucose Dulbecco's modified Eagle medium (DMEM, Gibco) containing 10% FBS and 1% Penicillin/Streptomycin (P/S) until reaching the total number of cells needed (1×10^6 cells/disc), in general in passages P4 to P8.

Intervertebral disc isolation

Bovine IVDs were isolated from young adult animals' tails (5–10 months old) within 3 hours post-slaughter in a local slaughterhouse (Carnes Landeiro, Barcelos, Portugal). All experiments were performed in accordance with relevant guidelines and regulations, with the ethical approval of the Portuguese National Authority for Animal Health. IVDs with CEPs were harvested in sterile conditions following a protocol previously described27. Briefly, the caudal discs with CEPs were removed using a band saw (Dremel ® Moto-Saw (MS20-1/5)) to obtain parallel cuts. The CEPs were afterward jet-lavaged with sterile phosphate-buffered saline solution (PBS, pH 7.4), using a Pulsavac wound debridement irrigation system (Zimmer, Inc., Switzerland). Discs were washed sequentially in 1%/10%/1% of P/S in PBS (pH 7.4) for 1/10/1 min, respectively. Afterwards, discs were incubated overnight in 6-well plates with high-glucose Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 2% fetal bovine serum (FBS, Gibco), 1% P/S (Gibco), 1% insulin transferrin selenium supplement (ITS) (BD, Becton Dickinson) and 0.1% Primocin (Invivogen) at 37°C in a 5% CO_2 atmosphere.

Intervertebral disc Culture

A previously described model of IVD nucleotomy accessed through the CEP was adopted to mimic the loss of ECM by the removal of part of the NP[27,30]. Briefly, a circular cavity was made in the CEP. After a portion of the CEP was removed, part of the NP (0.05–0.1 cm^3 of tissue) was removed using a blade. The removed CEP was afterwards repositioned, sealed with bone cement (PMMA, Vertecem V Cement Kit, Synthes, Switzerland). Finally, all the discs

were turned to place the cavity at the bottom of the well and incubated in medium (DMEM 4.5 g/L glucose, 2% FBS, 1% P/S, 1% ITS and 0.1% Primocin) in 6-well plates for 2 h before cell seeding. In these setup, two experimental groups were defined, the "cavity" disc with an empty cavity and the "C + hMSCs" disc, a cavity disc with seeded hMSCs on the CEP (Figure 1). Intact discs (discs without cavity) were used as a control group.

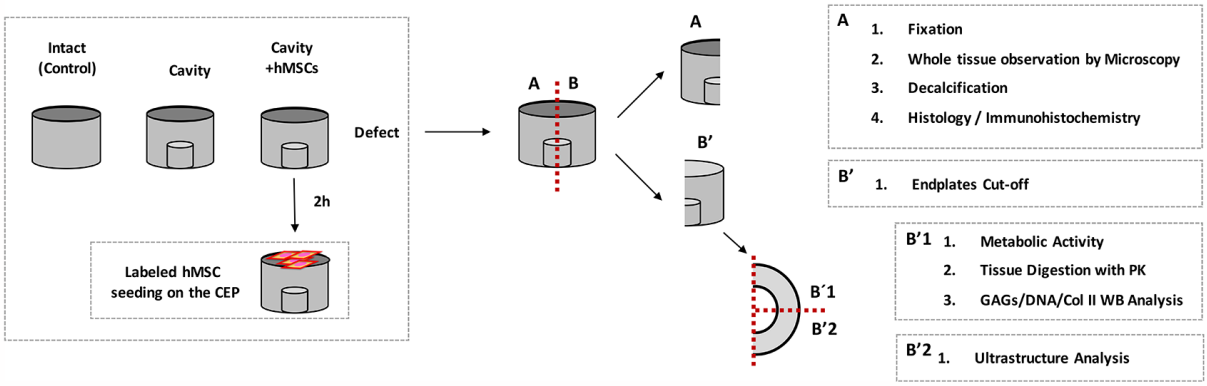


Figure 1 | Schematic representation of the experimental setup.

hMSCs were seeded in the C + hMSCs group, at a density of 1×10^6 cells/disc[27], on top of the CEP (i.e. on the side opposite to the injury) and incubated for 30 min to allow cells to enter the CEP structure. Afterwards, medium (DMEM 4.5 g/L glucose, 2% FBS, 1% P/S, 1% ITS, 0.1% Primocin) was added and discs were cultured at 37°C, in 5% CO_2 atmosphere, during 21 days. Media were exchanged every 2 days. After this period, discs were extensively washed with PBS and cut transversally with a blade, or sagittaly (with CEP) using a Dremel® Moto-Saw (MS20-1/5). Discs were then stored at −20 °C or fixed in 4% buffered formalin for 3–4 days, for further analysis. Independent experiments were performed using hMSCs from two different donors (n = 2) and ten animals (n = 10). An additional condition (n = 3) using labeled hMSCs was used for cell tracking after 21 days in the tissue. hMSCs were previously labeled with CellTracker™ CM-DiI Dye (Thermo Fisher Scientific Inc.) following the manufactures' instructions. The whole IVD sagittal section (Figure 1A) was imaged by high-content analysis system using In Cell Analyzer 2000 (GE Healthcare Life Sciences). Discs with non-labeled hMSCs were used as control.

Intervertebral disc Culture: metabolic activity, viability and cell proliferation analysis

Cellular metabolic activity of the disc tissue was measured using resazurin sodium salt assay as previously described by others[71,72]. Briefly, at day 21, discs were cut in half (Figure 1B) and the CEP removed in one of the half (Figure 1B′), remaining the AF and NP. This half was further divided in ¼ (Figure 9B′1) and incubated in a 6-well plate, with 10 mL of 10% resazurin solution (0.1 mg/mL resazurin sodium salt ($C_{12}H_6NNaO_4$, Sigma) in PBS) in DMEM

4.5 g/L glucose, 2% FBS, 1% P/S, 1% ITS, 0.1% Primocin during 3 h, at 37°C in a 5% CO_2 atmosphere. Relative Fluorescence unit (RFU) was measured at an excitation wavelength of 530 nm and an emission wavelength of 590 nm using a microplate reader, Synergy™ Mx multi-mode microplate reader (BioTek® Instruments, Inc., Vermont, CA). Cell metabolic activity was expressed as RFU normalized to DNA. A blank control comprising only medium was also included.

Cell proliferation was detected by IF, using Ki67 proliferation marker. Masked epitopes were exposed by treatment with 10 mM sodium citrate (pH 6) for 35 min at 95–98 °C. Paraffin sections were incubated overnight (4°C) with rabbit anti-Ki67 primary antibody (ab15580, Abcam plc, 330 Science Park, Cambridge CB4 0FL, United Kingdom, 1:100). Alexa Fluor 488-labeled goat anti-rabbit was used as a secondary antibody. All sections were mounted in Fluorshield with DAPI (Sigma). Control sections for each immunolabeling excluded primary antibody staining. Representative images were taken in an inverted fluorescent microscope (Axiovert 200M, Zeiss) with 40x and 100x objectives. The number of proliferative cells (Ki67+) was assessed for each group and counted manually on ImageJ 1.43u software (Wayne Rasband, National Institutes of Health, USA).

DNA Quantification

DNA content in the IVD tissue was quantified using a CyQuant® kit (Invitrogen). Briefly, 100 mg of NP tissue previously frozen at day 21 were minced into very small pieces and then digested in a proteinase K solution (0.5 mg/mL) overnight at 56 °C. DNA content was expressed in µg according to standard and normalized to the wet weight (mg) of the digested tissue.

Histology

IVDs with CEPs were harvested after 21 days of culture and fixed in 4% buffered-formalin. For haematoxilin & eosin (H&E) and safranin O-Fast green (SO-FG) staining, discs were cut sagittaly with CEPs and decalcified with 17% neutral ethylenedinitrilo-tetraacetic acid trisodium salt (EDTA, pH 7.0) for 10 days (EDTA solution was changed twice), and afterwards embedded in paraffin. Paraffin sections were cut in the sagittal plane at 3 µm. H&E was performed for an overall assessment of the histological structure. Sections were incubated in Gill's haematoxilin (Sigma-Aldrich) for 5 min to stain the cell nuclei, washed and dehydrated through graded alcohol solutions previous to a counterstain in alcoholic eosin (Surgipath) for 1 min, to detect cell cytoplasm and most connective tissue fibers. SO-FG was performed for assessment of matrix deposition in the tissue. Briefly, sections were incubated in Gill's haematoxylin (Sigma-Aldrich) for 5 min to stain the cell nuclei and, afterwards, immersed in 0.4% FG (Sigma) solution during 5 min to stain the collagen. After washing twice in 1% acetic

acid solution, slides were immersed for 4 min in 0.1% SO (Sigma-Aldrich) solution to detect proteoglycans deposition. Sections were imaged with an Olympus CX31 light microscope equipped with a DP-25 camera (Imaging Software CellˆB, Olympus, Center Valley, PA, USA) at different magnifications (10x, 40x and 100x).

Sulphated Glycosaminglycans quantification

GAG content in the IVD tissue was assessed in Proteinase K digested IVD tissue, as previously described for DNA quantification. GAG were quantified by the reaction with 1,9-Dimethyl-Methylene Blue zinc chloride double salt (DMMB, Sigma-Aldrich) dye reagent solution, containing 40 mM sodium chloride (NaCl, Roth), 40 mM Glycine (Roth) and 46 µM DMMB, previously adjusted to pH 3.0 as described71. Chondroitin sulphate A sodium salt from bovine trachea (Sigma) was used as standard. GAG content was expressed as µg/mg (wet weight) according to the standard. Results were normalized and presented as a fold increase to the cavity disc (n = 4).

Collagen type II quantification

Col type II quantification in the IVD tissue was assessed by western blot (WB). Briefly, part of previously frozen NP was minced and incubated with an optimized buffer for protein extraction containing 4 M guanidine hydrochloride (Sigma), 3 M sodium acetate (Merck) and 10 mM EDTA, and enriched with a protease and phosphatase inhibitor cocktail (Roche Diagnostics GmbH, Manheim, Germany and Sigma, respectively). Protein quantification was performed using the 2-D Quant Kit (GE Healthcare) according to the manufacturer's instructions. Protein samples (20 µg) were afterwards separated by sodium dodecyl sulphate (SDS)/9% polyacrylamide gel electrophoresis, and electroblotted onto a Hybond enhanced chemiluminescence (ECL) membrane (Amersham BioscienceseGE Healthcare). The monoclonal antibody against Col type II (1:1000 dilution) (II-II6B3) was used with a sheep anti-mouse (1:3000 dilution; Amersham Biosciences) horseradish peroxidase-conjugated secondary antibody, followed by ECL detection (Amersham Biosciences). Bands were quantified using Quantity One® 4.6.6 Software (Bio-Rad, Amadora, Portugal). Values were normalized to the density of each corresponding complete lane (total protein loaded)73. Results were afterwards normalized to the cavity group and presented as a fold increase (n = 7).

Immunohistochemistry

For IHC and IF techniques, non-decalcified IVD paraffin sections without CEPs were used. Agg and Col type I expression in the different IVD regions (NP and AF) was assessed by IHC. NovolinkTM Polymer Detection Kit (Leica Biosystems, Newcastle, UK) was used,

following the manufacturer's instructions. Antigen retrieval was performed through the incubation with a 20 µg/mL proteinase K (Sigma-Aldrich) solution for 15 min at 37 °C. For Col type I sections were boiled in citrate buffer (pH 6.0), as pre-treatment and then incubated with 1.5 U/mL solution of hyaluronidase (Sigma-Aldrich). After neutralization of endogenous peroxidase using Peroxidase Block for 5 minutes, a blocking step was performed. Sections were afterwards incubated with the primary antibody Agg (H-300) sc-25674 (Santa Cruz Biotechnology, Inc, Texas, USA) (1:50) or Col type I primary antibody (1:100) 600-401-103-0.1 (Rockland Immunochemicals, Inc. Limerick, PA), overnight. Bound antibodies were revealed after a 30 min incubation with NovolinkTM Polymer in the dark and 5 min incubation with peroxidase-substrate solution DAB. A negative control was performed in each slide without the primary antibody. Representative images of the slides were taken using an Olympus CX31 light microscope (20x objective for counting and 100x oil objective for detailed imaging of Agg IHC; 10x for Col I IHC). Agg and Col type I matrix staining in the different regions of the IVD (NP and AF) was qualitatively assessed by two independent observers. Agg cellular staining was quantified using a custom-made software written in MATLAB (The MathWorks Inc., Natick MA, USA) named ImmunoCellCount (see Supplementary Data) (n = 5).

Col type II distribution was analyzed by IF staining. For IF, antigen retrieval was performed in paraffin sections through incubation with a 20 µg/mL proteinase K (Sigma-Aldrich) solution for 15 min at 37 °C. After a blocking step, sections were then incubated for 2 h at 37 °C with the primary antibody against Col II (1:50) (monoclonal antibody against Col type II (II-II6B3) developed by Dr. Thomas F. Linsenmayer, from the Developmental Studies Hybridoma Bank under the auspices of the NICHD and maintained at the University of Iowa, Department of Biology, Iowa City, IA 52242). Alexa Fluor 594-labeled goat anti-mouse (Invitrogen-Molecular Probes, 1:1000) was used as the secondary antibody for Col II detection during 1h, room temperature, in the dark. All sections were mounted in Fluorshield with DAPI (Sigma). Control sections for each immunolabeling excluded primary antibody staining. Representative images were taken using an inverted microscope, Axiovert 200 M, Zeiss. Col II intensity in the different regions of the IVD (NP and AF) was quantified using a custom-made software written in MATLAB (The MathWorks Inc., Natick MA, USA) named IntensityStatisticsMask (see Supplementary Data) (n = 5).

Ultrastructure Analysis by Transmission electron microscopy (TEM)

Previously fixed portions of IVD tissue from each experiment were further processed for TEM. The different IVD regions, AF and NP were separated and cut into very small pieces. These samples were washed in PBS and fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate (pH 7.4) for 2 h. After washing in 0.1 M sodium cacodylate buffer for 30 min the tissue was fixed in 2% (v/v) osmium tetroxide in 0.1 M sodium

cacodylate overnight followed by another fixation with 1% uranyl acetate overnight. Samples were dehydrated in gradient series of ethanol solutions as follows: 50% ethanol for 10 min, followed by 70%, 80%, 90%, 96%, 100% and propylene oxide (v/v). Inclusion in EPON resin was performed by immersion of tissue in a gradually increasing series of propylene oxide to EPON as follows: 2:1, 1:1, 1:2 and 0:1 for 60 min each. At the end, inclusion of the tissue in EPON resin was performed in a silicon mold. EPON polymerization took place at 60 °C for 48 h. Sections with 50 nm thickness were prepared using a diamond knife (Diatome, Hatfield, PA, USA) and were recovered to 200 mesh Formvar Ni-grids. Staining of sections using 2 wt% uranyl acetate and saturated lead citrate solution, for 7 min each, was performed before observation. Visualization took place at 80 kV in a (JEOL JEM 1400 microscope (Japan)). Both AF and NP from the different bovine donors were analyzed per group (cavity and C + hMSCs), and a freshly isolated disc was used as a control. 20–30 images for each sample were analyzed by two independent observers (n = 3). A qualitative analysis of several parameters was performed based on previous studies46 and categorized by us in a scoring system (Table 1) in what concerns to: i) lamellar organization of the AF (highly organized (++++), disorganized (+)); ii) matrix density (higher matrix density (++++), sparse matrix (+)); iii) collagen fibers size heterogeneity (from high to low). The cellularity of the tissue, i.e. the presence of cells and their activity (very active, synthetic activity or necrotic) was also accessed (see Supplementary Data).

Picrosirius-polarization method and quantification of birefringent fibers

The picrosirius-polarization method and consequent birenfrigent fibers quantification was performed in IVD tissue sections to assess the structural changes based on the birefrigence of the collagen fibers48. Sections of 3 μm were simultaneously stained with Sirius Red solution for 1h to avoid variations. Picrosirius Red stained sections were analyzed through a polarizing lens and all images were captured with the same parameters. A color threshold was applied using ImageJ software (version: 148r) in order to identify and quantify red, yellow and green fibers (n = 3).

Growth factors Analysis in IVD culture media

A commercially available array of 42 growth factor proteins (RayBio® C-Series Human Growth Factor Antibody Array C1, #AAH-GF-1-4, RayBiotech, Inc. 3607 Parkway Lane, Suite) was used to evaluate the relative levels of growth factors production in the IVD culture media of both cavity and C + hMSCs at day 21. A pool of culture media collected from 6 independent experiments was prepared for this assay, and 1 mL of the prepared pool was used. The array was performed by following the manufacturer's instructions. Data shown represents 5 min exposure in Chemidoc XRSþ (BioRad). Results were generated by quantifying the mean spot

pixel density from the array using image software analyses (ImageLab 4.1; BioRad). The densities of signals obtained were normalized with the background and results presented as a mean of two spots per growth factor.

Statistical Analysis

Statistical analysis was conducted using GraphPad Prism version 6.0f for Mac OS X (GraphPad Software, California, USA). The non-parametric Mann-Whitney test was used to compare two groups of non-related samples. Statistical significance was considered whenever $*p < 0.05$; $**p < 0.01$; $***p > 0.001$ and when $p \geq 0.05$ as not significant (NS).

RESULTS

IVD long-term organ culture: metabolic activity, cell proliferation and hMSC migration

To investigate the effect of CEP-seeded hMSCs on the ECM remodeling of the IVD, whole organ cultures of nucleotomized discs from bovine origin were used as a model, similarly to our previous study[27]. hMSCs were seeded on the discs CEP and maintained in culture for 21 days. First, hMSC viability and survival in IVD culture media (supplemented with 2% Fetal Bovine Serum (FBS)) was confirmed by Annexin/Propidium Iodide staining (see supplementary data). After 21 days, the different conditions (control, cavity and C + hMSCs) were compared in terms of metabolic activity, DNA content and cell proliferation. Tissue/cells metabolic activity was evaluated by resazurin assay and showed a slight increase per cell in the cavity group (1021 ± 616 RFU/µg of DNA), although no significant differences were observed when compared to control (813 ± 500 RFU/µg of DNA) and C + hMSCs group (628 ± 538 RFU/µg of DNA) (Figure 2A).

The DNA content of nucleotomized (0.03 ± 0.01 µg/mg) and control (0.04 ± 0.01 µg/mg) discs was similar, but a slight increase in the C + hMSCs group was observed (0.06 ± 0.04 µg/mg) (Figure 2B). Cell proliferation was additionally evaluated by Ki67 positive expression using immunofluorescence (IF). In control IVDs, 4 ± 2% $Ki67^+$ cells were found, while a slight increase in cell proliferation was observed in both cavity (10 ± 2% $Ki67^+$ cells) and C + hMSCs groups (9 ± 4% $Ki67^+$ cells) (Figure 2C,D). Although a tend to increase in cell proliferation was observed in both nucleotomized groups, the values were out of statistical differences. The presence of hMSCs in the IVD tissue at day 21 was confirmed in a mosaic image of the whole IVD obtained by a high content screening system (InCell Analyzer 2000), in which CM-DiI red-labeled hMSCs could be identified (Figure 2E-a,E-b (white arrows)). A control IVD with non-labeled hMSCs was also imaged (Figure 2E-a′b′). These results showed that the majority of hMSCs remained on the CEP (red fluorescence), and a small proportion migrated throughout the tissue (about 3%, estimated by quantification of a central sagittal section of the IVD).

Since hMSC migration is mostly related with metalloproteinases (MMPs) production, MMP2 and MMP9 present in the organ culture media were evaluated by gelatin zymography. This technique allows for the detection of both active and inactive forms of MMPs. The results showed a higher activity of MMP2, both pro- and active form, in the C + MSCs group, while MMP9 activity was absent (see Supplementary Data). Nevertheless, the cavity group (without hMSCs) also revealed higher amounts of MMP2, so it was not possible to conclude whether MMP2 was produced by hMSCs or IVD cells, stimulated by the presence of hMSCs.

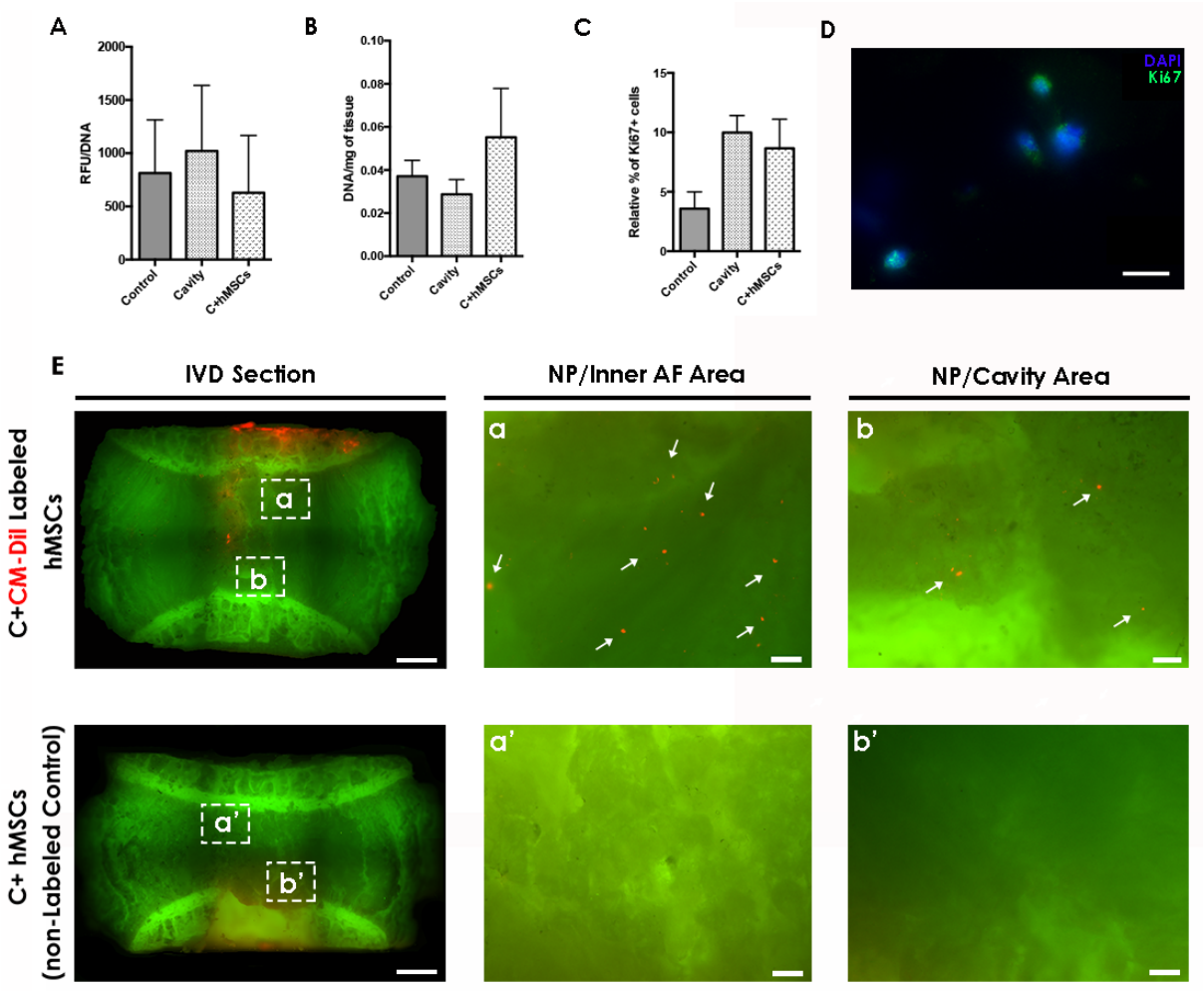


Figure 2 | Metabolic activity, DNA, Cell proliferation and hMSCs migration in the IVD tissue after 21 days of culture. Control, cavity and C + MSCs were compared at day 21 for: **A |** metabolic activity, no differences were observed between the groups; **B |** DNA content, a slight increase in DNA was observed in the C + hMSCs group; **C |** cell proliferation quantification: higher cell proliferation was observed in both lesioned groups (cavity and C + hMSCs). All depicted results are presented as M/D (n = 3-7) without significant differences (Mann-Whitney test). **D |** representative image of proliferative Ki67+ cells in the IVD tissue is presented (scale bar: 10 µm). (E) representative image of CM-Dil-labeled hMSCs and the control with non-labeled hMSCs after 21 days in IVD organ culture (scale bar: 2000 µm / magnified image: scale bar: 200 µm). CM-Dil labeled hMSCs can be identified in red in the IVD tissue at day 21.

Histological analysis of the IVD after long-term culture

The impact of hMSCs seeded on CEP in nucleotomized IVD after 21 days of culture was first evaluated by histology using hematoxylin/eosin (H&E) and safranin-O fast-green (SO-FG) staining. Representative sagittal sections of decalcified discs are depicted in Figure 3. H&E staining allowed the observation of different and interrelated areas of the IVD, the CEP on the top and bottom of the IVD, the IVD central part (NP) and the surrounding fibrous, AF (Figure 3A). A higher cell density was observed in the AF when compared with the NP area. Typical cells of the AF ('fibroblast-like') and NP ('chondrocyte-like') could be distinguished, as highlighted by the black arrows. At day 21, it was difficult to observe the lesion area as a result of tissue swelling (Figure 3A,B). Nonetheless, when comparing tissue morphology in different conditions, a more organized structure was observed in control discs, while in the lesioned groups (cavity and C + hMSCs), namely in the NP, ECM fibers appeared to be more wrinkled, ruptured and disorganized. SO-FG staining allowed the distinction between peripheral AF (in blue), rich in collagen, and the inner AF and NP, containing mostly proteoglycans (in orange) (Figure 3B). AF structure was maintained in all groups. The tenuous orange staining observed in the NP area of the cavity group, suggested the loss of proteoglycans, possibly as a result of the injury process. On the other hand, a stronger orange staining in the C + hMSCs group suggests for higher proteoglycan deposition in the NP (Figure 3B). Furthermore, some areas of intense bluish-green were identified in the NP, indicating the presence of collagens (Figure 3B, magnified image, highlighted by the arrows). The presence of collagen staining in the NP (bluish-green areas) increased with hMSC treatment. Moreover, we excluded the presence of calcifications resulting from hypothetical hMSC differentiation into osteogenic lineage by alizarin staining (seeSupplementary Data).

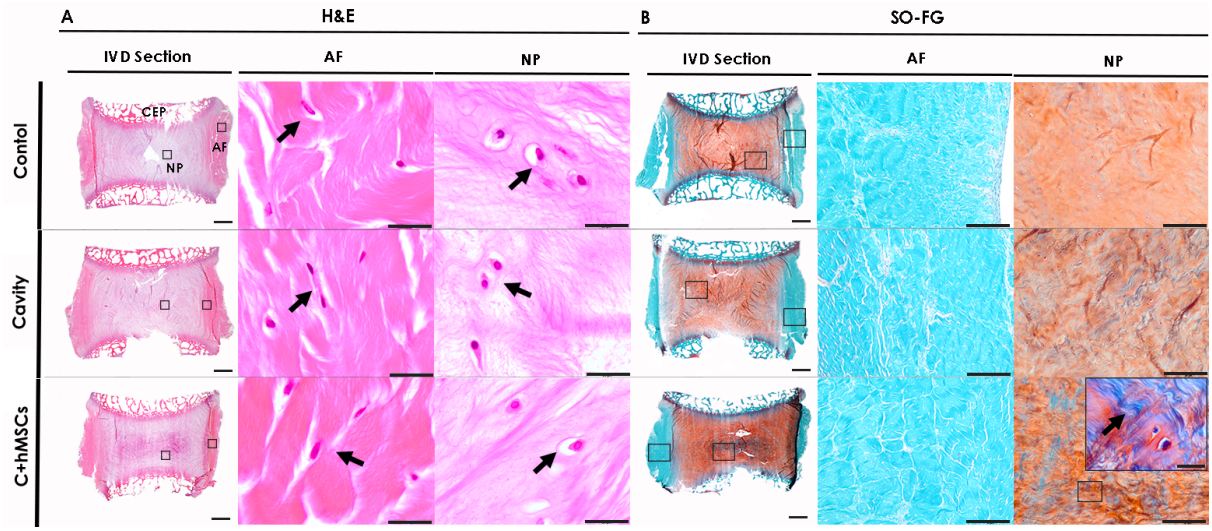


Figure 3 | Histological analysis of the IVD tissue after 21 days of culture. Sagittal sections of the control, cavity and C + hMSCs were stained with **A |** H&E and **B |** SO-FG. One representative donor was depicted. In the IVD whole section (scale bar: 2000 µm), CEP, NP and AF regions of IVD can be distinguished. Magnifications of NP/AF cells are presented (scale bar: 20 µm) in the H&E staining as well as magnifications of the whole AF/NP tissue in SO-FG (scale bar: 200 µm). Collagens are visualized by green/blue (arrow) color while GAG are observed in orange color. A higher intensity of orange color in the C + hMSCs group NP suggested a higher proteoglycan content in the tissue of this experimental group.

Sulphated Glycosaminoglycans and Collagen type II quantification in the IVD tissue

Sulphated Glycosaminoglycans (GAG) and Col type II were also analyzed in the NP of IVDs in the different groups, upon tissue digestion. GAG content was similar between all the groups (Figure 4A) and no significant differences were observed. Col type II, quantified by western blot (WB), a slight increase in the C + hMSCs group was observed, but no significant statistical differences were detected compared with the control and cavity groups (Figure 4B).

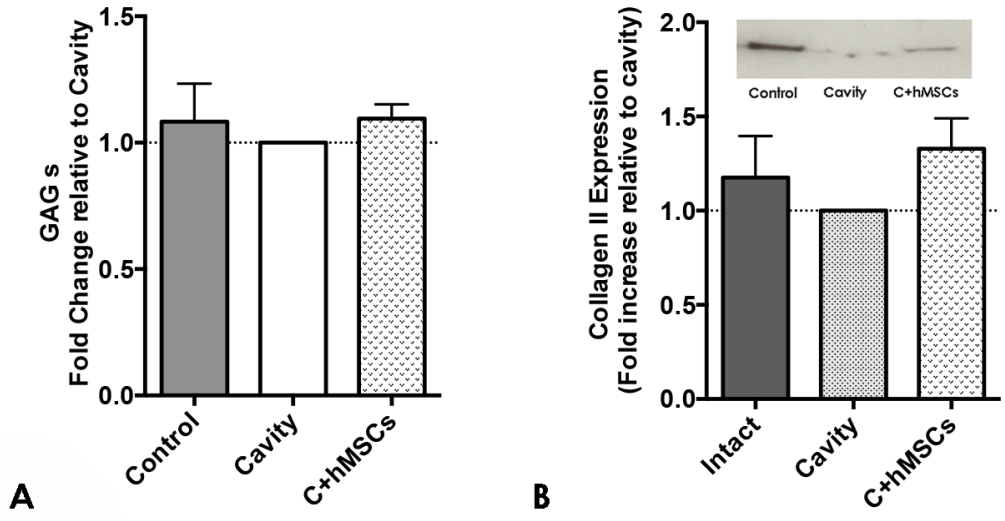
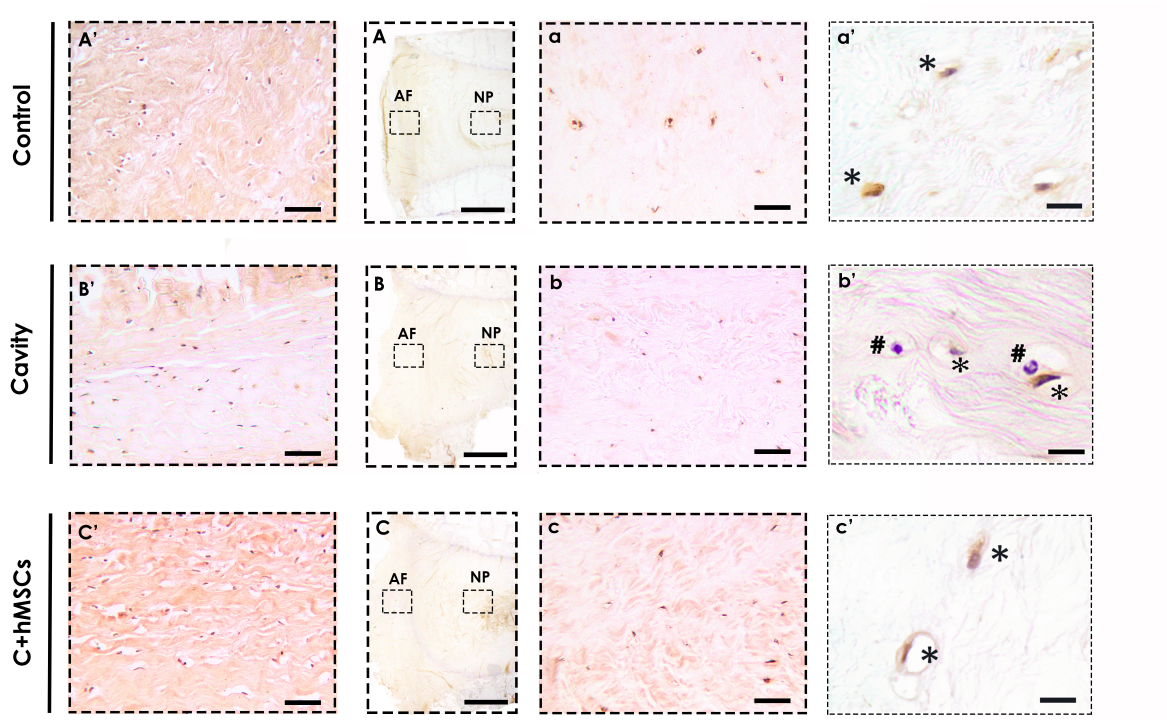


Figure 4 | GAG and Collagen type II quantification in the NP tissue at day 21. The NP tissue of the three experimental groups was digested and afterwards analyzed for GAG and Col type II content. (A) Fold increase in GAGs content. GAG content was similar between the groups (n = 4). (B) Fold increase in Col type II and a representative image of a blot. Col type II content was determined by WB and values normalized to the total protein and presented as fold increase to the cavity group; a slight increase in Col type II was observed in the C + hMSCs group compared with the cavity (no statistically significance in raw data, Mann-Whitney test (n = 7)). Results are presented as M/D.

Aggrecan expression in the IVD tissue

The expression and distribution of aggrecan (Agg) (the most abundant proteoglycan) in the IVD tissue was investigated *in situ* using immunohistochemistry (IHC) (Figure 5A). Agg displayed both matrix and cellular staining after 21 days in culture in the IVD tissue (Figure 5A–C). Concerning matrix staining, Agg expression was more intense in the AF (Figure 5A′–C′) when compared with NP (Figure 5a–c). In addition, a higher Agg expression was observed in both control and C + hMSCs groups in comparison to the cavity, particularly in NP where the differences were more accentuated (Figure 5D). Regarding the Agg cellular staining, this was associated with a localized protein accumulation in the cell surroundings or even cell co-localization. Agg + cells adopted a brownish (*) color while negative cells were purplish (#), allowing distinction between the two classes (Figure 5a′–c′). The total number of cells and the number of Agg+ cells were determined (Figure 5E) using a custom-made software ImmunoCellCount (Figure 5F) (see Supplementary Data). First the manual and automatic quantification of total number of cells were compared and no significant differences in the number and percentage of Agg+ cells, in both AF and NP was observed (Figure 5G,H). Nevertheless, the time required for manual versus ImmunoCellCount-assisted calculation of Agg+ cells was longer, taking about 4.2 times more counting cells manually than with the program (Figure 5) (***$p < 0.001$). Still, the software presents some limitation in the identification of elongated cells, which can be easily overcome with the manual addition tool or by alteration of the cell size/threshold parameters. Moreover, false positive/negative counts can occur. Nevertheless, we estimate that the frequency these errors is around 5%, which encourages the use of the software to count Agg+ cells in IHC sections.

The frequency of Agg+ cells was slightly higher in the NP of control discs, comparing to the AF (52 ± 30% vs 35 ± 18%, respectively). A decrease of Agg+ cells was observed in cavity discs both in the AF (19 ± 13%) and NP (28 ± 16%). In the presence of hMSCs, the proportion of Agg+ cells increased in the AF (37 ± 30%) and was significantly higher (*$p < 0.05$) in the NP (77 ± 8%), when compared with the cavity group (Figure 5E).

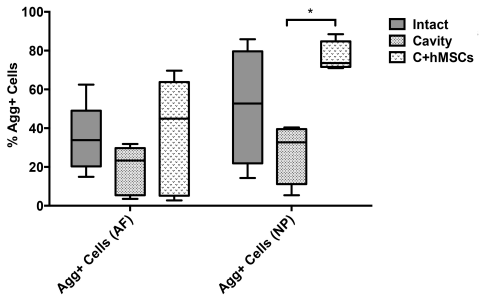


D

Qualitative analysis of Agg expression by IHC in the IVD matrix.

	Control	Cavity	C+hMSCs
AF	++++	+++	++++
NP	+++	+	+++

E



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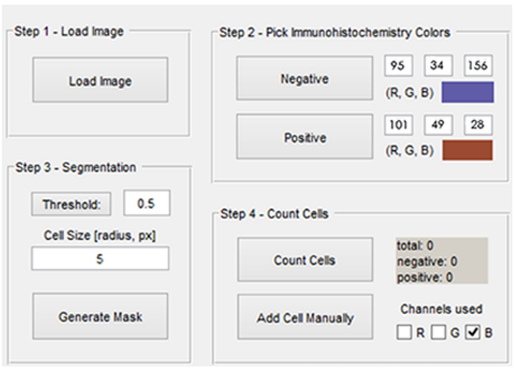

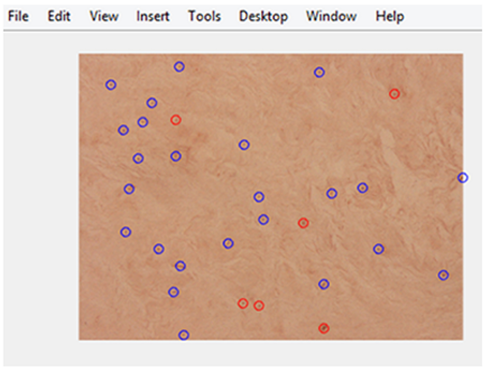

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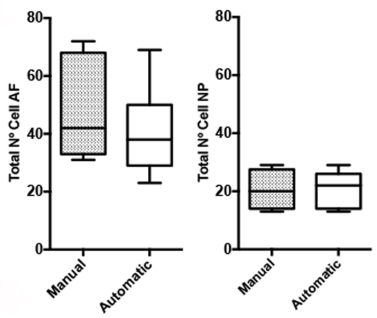

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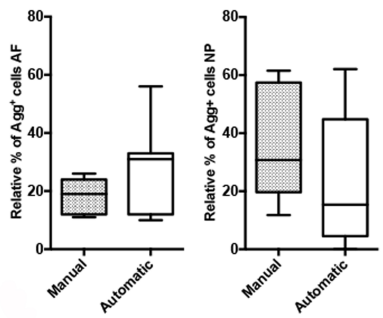


I

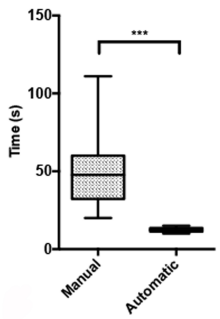


Figure 5 | Aggrecan expression and quantification in the IVD tissue at day 21. Agg expression in the tissue was evaluated by IHC and displayed both matrix and cellular staining. **(A–C)** Overall Agg expression in the tissue. One representative donor is presented (scale bar-2000 μm). **(A′–C′)** Magnified representative images of Agg matrix and celular expression in the AF area (scale bar-200 μm). **(a,b)** Magnified representative images of Agg matrix and celular expression in the NP area (scale bar-200 μm). **(a′–c′)** High magnification images of cellular Agg expression in the NP (scale bar 20 μm). **(D)** Qualitative analysis of Agg matrix expression both in the AF and NP area. Cavity group displayed a less Agg matrix expression when compared to the control and the C + hMSCs group. **(E)** Quantification of Agg cellular expression (Agg+ cells were quantified using ImmunoCellCount software). Agg+ cells could be distinguished by a characteristic brown color, in co-localization with the hematoxylin purple staining. A significant reduction of Agg expression is observed in the cavity group in both AF and NP areas, while a significant increase is observed in the presence of hMSCs. Results are presented as box-and-whiskers plots (n = 5). **(F)**ImmunoCellCount software. Image on the left side, GUI where the settings can be established and on the right side, the image after the classification. Comparison between manual assessment of Agg deposition vs automatic using the ImmunoCellCount software was performed in both AF and NP, concerning: **(G)** Total number of cells; **(H)** Relative % of Agg+ cells; **(I)** Time of the analysis. No significant differences were observed between the two methods, but a there was a significant improvement in the time consumed for image analysis. Results are presented as box-and-whiskers plots (n = 5). Statistical analysis was performed using Mann-Whitney test (*$p < 0.05$; ***$p < 0.001$).

Collagen type II expression in the IVD tissue

Col type II expression and distribution *in situ* was also investigated in the IVD tissue using IF (Figure 6A) and quantified by fluorescence intensity (see Supplementary data). Col type II mean fluorescence intensity (MFI) in NP and AF was calculated separately. Col type II intensity in the AF was similar between all the groups tested: 25 ± 9 (control), 24 ± 5 (cavity) and 28 ± 7 (C + MSCs); while in NP, hMSCs significantly increased (*p < 0.05) the expression of Col type II (34 ± 11), when compared with cavity (23 ± 6) (Figure 6B).

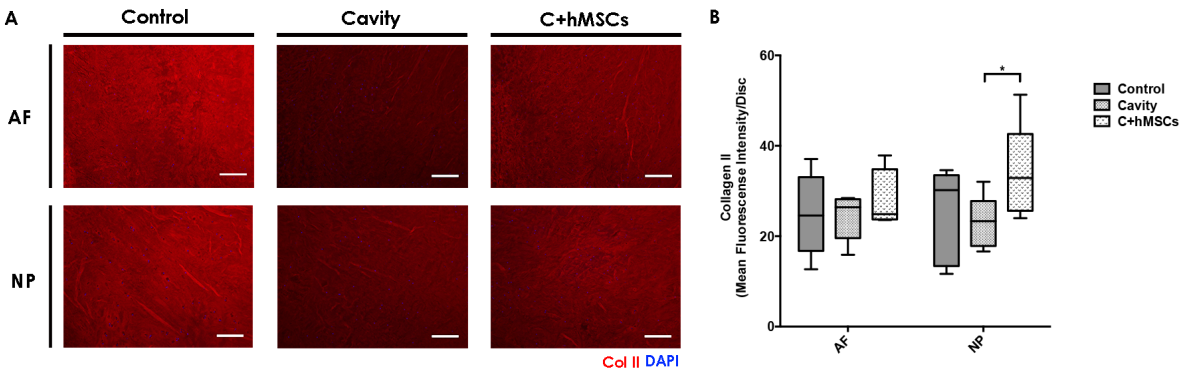
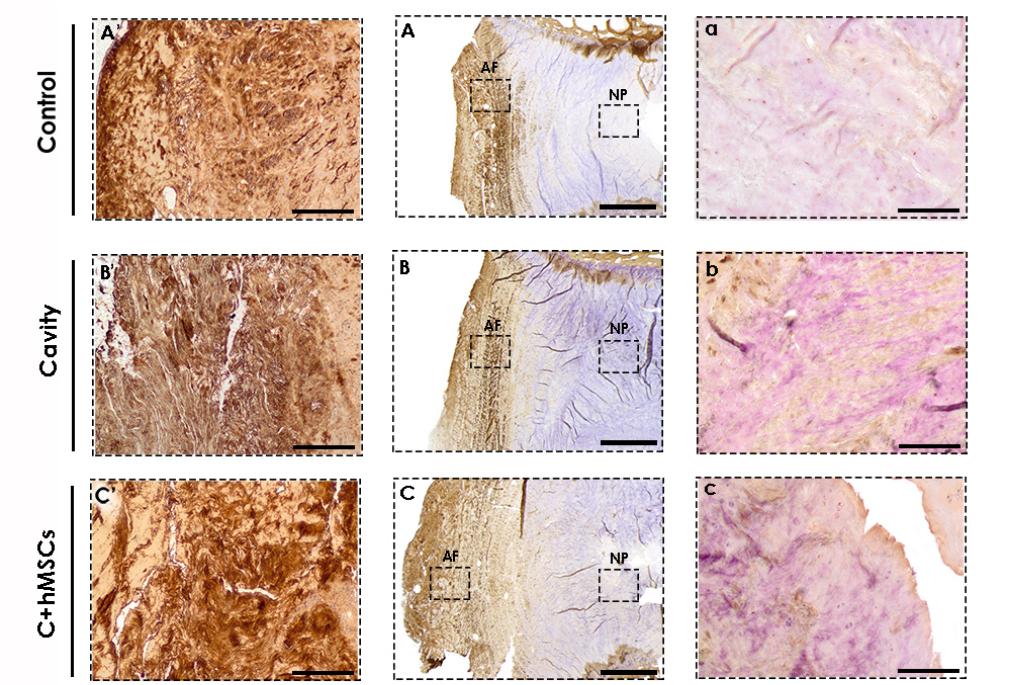


Figure 6 | Collagen type II expression and quantification in the IVD tissue. At day 21 sagittal sections of control, cavity and C + MSCs) were analyzed for Col type II expression and distribution by IF. **(A)** Col type II expression in the tissue. One representative donor is presented (scale bar-200 µm). **(B)** Col type II expression was quantified using IntensityStatisticsMask Software (see supplementary data). A significant increase in Col type II expression was observed in the presence of hMSCs, in the NP area. Results are presented as box-and-whiskers plots (n = 5). Statistical analysis was performed using Mann-Whitney test (*p < 0.05).

Collagen type I expression in the tissue

Col type I expression and distribution in the IVD tissue was also analyzed by IHC (Figure 7A–C) and qualitatively analyzed (Figure 7D). An intense expression of Col type I was found in the outer AF area decreasing gradually towards the NP central area. Overall the expression in AF areas was similar between the groups (Figure 7A′–C′), while in the NP area, an increase of Col type I expression was noticed in both groups with lesion (cavity and C + hMSCs) when comparing to the control (Figure 7a–c). Furthermore, no noticeable differences in Col type I expression were found in the NP of both cavity and C + hMSCs group.



D

Qualitative analysis of Col type I by IHC in the IVD matrix.

	Control	Cavity	C+hMSCs
AF	++++	++++	++++
NP	+	++	++

Figure 7 | Collagen type I expression in the IVD tissue. At day 21 sagittal sections of control, cavity C + MSCs were analyzed for Col type I expression and distribution by IHC. **(A–C)** Overall expression of Col type I in the IVD tissue. One representative donor is presented (scale bar-2000 µm). Col I expression was more pronounced in the outer AF area, decreasing towards the NP central area (n = 3). **(A′–C′)** Magnified images of the AF area (scale bar-500 µm); **(a–c)** Magnified images of the NP area (scale bar-500 µm). **(D)** Qualitative assessment of col type I expression in the IVD matrix. A Higher expression was observed in lesion groups (cavity and C + hMSCs).

***IVD tissue ultrastructure and collagen fiber quantification*.**

The ultrastructure characterization of the IVD was observed by transmission electron microscopy (TEM) allowing a more profound visualization of matrix/cell alterations that occur during the degenerative process. Both AF and NP ultrastructure of the IVD of each group were imaged after 21 days in culture (Figure 8A). A qualitative analysis of the ultrastructure was performed, detailing the most relevant parameters for AF and NP: lamellar organization, matrix density and collagen fibrils (Table 1, n = 3). In the control disc, namely in the AF area, a high organization of the collagen fibers was observed, and fibrils were orderly organized with a lamellar disposition. The collagen fibers were mostly homogenous. Also, a highly dense matrix was present in both AF and NP of the control disc. In the cavity disc, the lamellar organization of the AF fibers were partially lost, becoming more randomly distributed. There was also a significant loss of matrix in both the AF and NP area, and higher fiber heterogeneity in the cavity group. On the other hand, in discs treated with hMSCs (C + hMSCs), some lamellar organization was observed, as well as a more dense matrix in both AF and NP, more similar to the control group. In addition, the collagen fibers seem to be less heterogeneous, in both AF and NP, when compared to the cavity group. At the cellular level, both active and healthy cells and necrotic cells were found in all groups, although non-representative numbers of cells were observed. Healthy cells had signs of cell activity and matrix synthesis (normal cell nucleolus, intact cytoplasm and organelles; dense ECM matrix components encircling cells), while necrotic cells presented signals of chromatin clumping and, in some cases, cytoplasmatic deterioration (see Supplementary Data).

Collagen fibers in both AF and NP were further studied based on birefringence intensity using Picro-Sirius red staining (PSR). By this technique, the thick/mature fibers display an orange/reddish color under polarized light and the intermediate mature fibers display a yellow color (these two are often associated with Col type I), while the thin/immature fibers display a green color (generally associated with Col type III)[28]. Col type II, typically of cartilaginous tissues, such as the IVD, forms very small fibrils which are embedded in ground substance and therefore result in a weak birefringence of varying color[28]. A representative image of PSR staining can be observed in Figure 8B. The different colors of the collagen fibers (red, yellow and green) were observed by polarized light and quantified accordingly (Figure 8C). The green birefringence represented the smaller percentage of fibers, compared to yellow-reddish fibers in both AF and NP. The control disc, presented a balanced distribution of the three types of fibers. In the NP of the cavity and C + hMSCs groups, a higher percentage of mature fibers (red) was found, which might be explained by the expression of Col type I. By determining the ration of greenish/reddish fibers (Figure 8D) we observed a decrease in both NP and AF of the cavity group (NP,

0.04 ± 0.02; AF, 0.07 ± 0.1), compared with control discs (NP, 0.5 ± 0.1; AF, 0.2 ± 0.02). This ratio slightly increased in the NP of the C + hMSCs group (NP, 0.09 ± 0.06; AF, 0.07 ± 0.03) suggesting higher synthesis of new fibers and thus corroborating the increase in Col type II in the NP of C + hMSCs group.

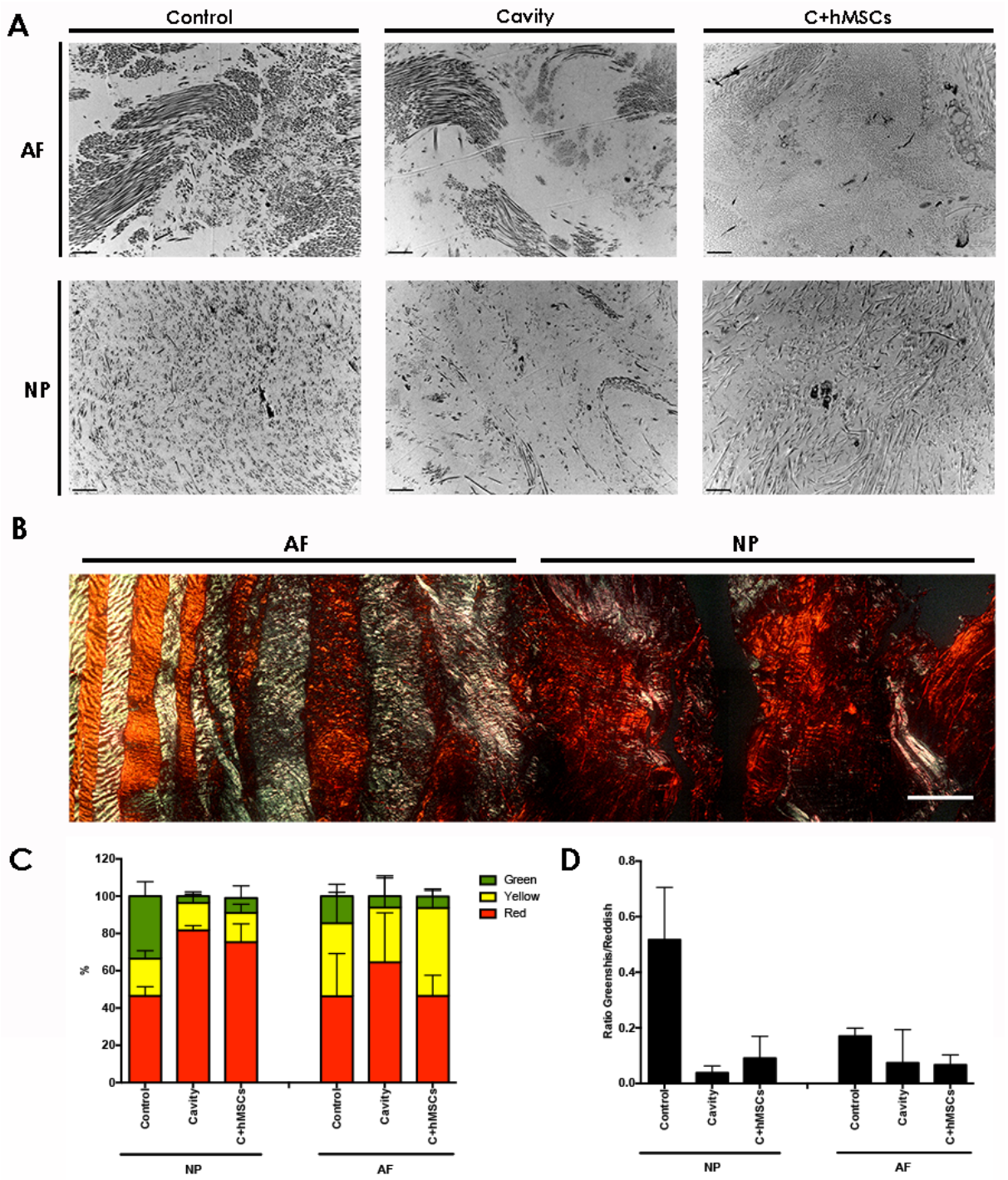


Figure 8 | IVD ultrastructure and Collagen Fibers quantification. At day 21, controls and both cavity and C + hMSC ultrastructure were analyzed by TEM. (A)One representative donor is presented (scale bar-2 μm). Images were analyzed and scored for the different parameters (Table 1, (n = 3)). A loss of lamellar organization as well as ECM matrix was observed in the cavity group. Density levels seem to be recovered in the presence of hMSCs. In both conditions, cavity and C + hMSCs, a higher variability in the Coll fibers was observed, suggesting ECM remodeling. (B) Histological analysis of IVD stained using picrosirius red and visualised by polarised light microscopy (representative image of 1 disc). (C) Quantification of the relative percentage of collagen fibers (n = 3). (D) Ratio greenish/reddish (mature/immature) collagen fibres. The ratio of greenish/reddish fibers was very similar in the AF area while in the NP, where the lesion was previously performed, this ratio decreased in the cavity group and slightly increased in the presence of hMSCs.

Table 1 | Qualitative analysis of the main features of IVD ultrastructure.

Parameters	**Lamellar Organization**	**Matrix Density**		**Collagen Fibers**	
IVD Area	**AF**	**AF**	**NP**	**AF**	**NP**
Control	**+++**	**+++**	**+++**	**Low Variation**	**Low Variation**
Cavity	**+**	**++**	**+**	**Moderate Variation**	**High Variation**
C+hMSCs	**++**	**+++**	**++**	**Moderate Variation**	**Moderate Variation**

Growth factor production in ex vivo IVD cultures with MSCs

A screening of growth factors production was performed, in an attempt to unveil the mechanisms underlying NP matrix remodeling by hMSCs seeded in CEP. Organ culture supernatants of nucleotomized IVDs (cavity) and IVDs with hMSCs on CEP (C + hMSCs) at 21 days of culture were pooled (n = 6) and analyzed by an antibody array of 42 growth factors (see Supplementary Data). Seven growth factors were observed to be up-regulated in the medium of the C + hMSCs group while only one was up-regulated in the cavity group (Figure 9A,B). However, it was not possible to distinguish between bovine or human proteins. The presence of hMSCs (C + hMSCs) considerably increased the levels of: i) fibroblast growth factors-family, FGF-6 and FGF-7, insulin-like growth factor binding protein 6 (IGFBP6) and Stem cell factor (SCF) (>6); ii) insulin-like growth factor 1 receptor (IGF-1 sR) (>4); iii) placental growth factor (PLGF) and granulocyte macrophage colony-stimulating factor (GM CSF) (>2). In addition, the presence of hMSCs decreased the levels of platelet-derived growth factor receptor, alpha (PDGF R alpha) compared to the cavity group (<0.5) (Figure 9C).

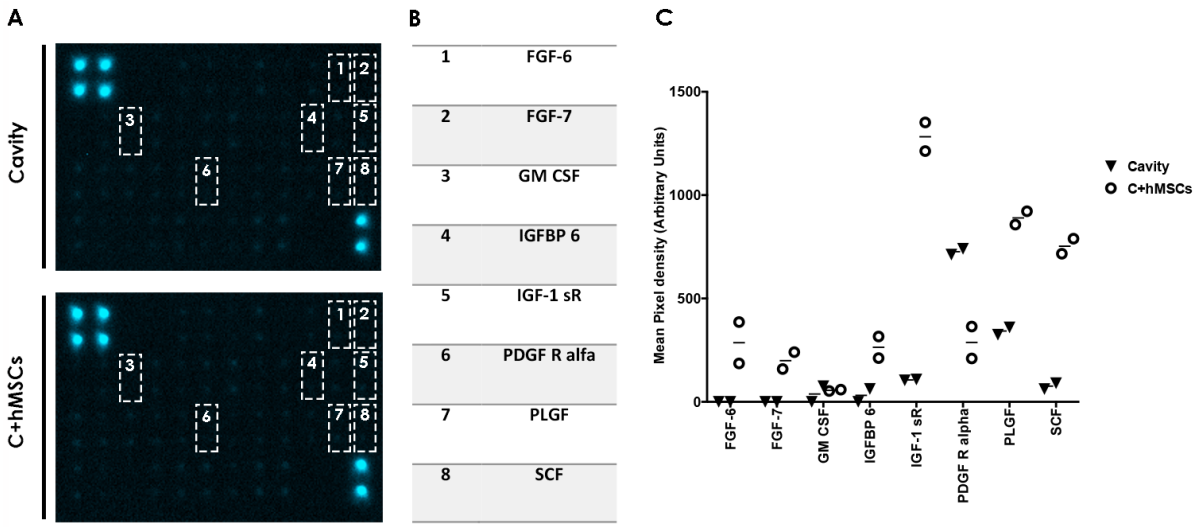


Figure 9 | Growth factor production in the IVD culture. Growth factors production was evaluated using an array, in a pool of 6 donors. (A) Images of the array membranes obtained for each experimental group (cavity and C + hMSCs); (B)Table with the key detected cytokines. (C) Quantification of the key growth factors by quantifying the mean spot pixel density from the array. The densities of signals were normalized with the background. Results are presented as a plot of individual values (two dots). 7 key GFs were shown to be increased in the presence of hMSCs, while one of them decreased. In general, all the key GFs were directly or indirectly related with cartilage remodeling and ECM synthesis.

DISCUSSION

MSCs are one of the most attractive cell types for IVD regeneration, as recently reviewed by Vadalà *et al*.[29]. Among the different sources of adult stem cells, including adipose, muscle or even IVD-derived stem cells, bone marrow (BM) derived MSCs are by far the most used source of cells. BM-MSCs have shown great differentiation capacity towards NP cells phenotype within multiple studies and have already been used in clinical trials, although the access to BM may entail risks and side effects. Nevertheless, adipose- and muscle-derived stem cells efficacy has not been established yet and IVD-derived stem cells are poorly characterized, lacking standardization of isolation methods and involve several risks in the harvesting procedure[29].

The injection of hydrogel-encapsulated MSCs in the cavity of nucleotomized discs revealed that MSCs remain viable and are able to differentiate towards a disc-like phenotype[30]. Still, MSC administration via CEP aims to overcome the difficulties often associated with hydrogel injection in the NP (extrusion and cell leakage) in cell transplantation approaches, which are commonly performed via the AF and might lead to its rupture[16,17]. MSCs are able to migrate to injured tissues and act either on cell "replacement" (multilineage differentiation capacity) or on cell "empowerment" (release of immunomodulatory factors and/or growth factors)[31]. MSC migration towards the IVD was first reported by Junger *et al*.[26] using an *ex vivo* model cultured under stimulated-degenerative conditions. Recently, using an *ex vivo* model of nucleotomized disc, we showed that hMSCs seeded on CEP could migrate towards the NP, and are additionally enhanced by SDF-1 incorporation into a hydrogel[27]. The advances in surgical techniques to access the NP avoiding AF damage, opened new perspectives for cell transplantation. Vadalá *et al*.[19,20] reported a surgical transpedicular approach via the CEP as an alternative route for IVD regenerative strategies, in a sheep model.

The present study focused on the particular effect of hMSCs seeded on CEP in the IVD ECM remodeling. For that, an *ex vivo* model of nucleotomized IVD with CEP was used, which allows the study of IVD degeneration and regenerative strategies in a controlled manner, maintaining an intact AF[30], while preserving the IVD morphology with the CEP. This model was previously used and maintained in culture for 2 days by our group[27] and up to 7 and 14 days in culture by others[30,32]. In this study, long-term culture periods were fundamental to understand repair/regenerative processes at the protein level. Nonetheless, IVD long-term culture is a challenge due to the balance between nutrient supply and tissue structure maintenance, i.e. in prevention of swelling. The presence of the CEP in IVD cultures was shown to maintain IVD structure and to favor tissue viability, metabolism and to impair swelling and matrix degradation[33,35]. In this study, our model was maintained for 21 days. After this period, metabolic activity, cell proliferation and DNA content was firstly assessed. Globally, all

the experimental groups were metabolically active, with low numbers of proliferative cells (<15%). Low cell proliferation rates appear to be common in both healthy and herniated human discs[36]. The $Ki67^+$ proliferative cells were more frequently found in the cavity and C + hMSCs groups, suggesting that the injury by itself might stimulate cell proliferation and could represent a matrix repair response to the stimuli triggered by the cavity. This response, triggered by a stimuli, was reported by Johnson *et al*.[37], in pathological human discs. The DNA levels were slightly higher in the hMSC treated group, suggesting higher cell content possibly due to cell migration from the CEP.

hMSC migration in the IVD tissue was confirmed after 21 days of culture, by detection of CM-DiI-labeled hMSCs (red) in both, AF and NP regions, namely in the lesion (cavity) area. Cell migration in the ECM occurs via degradation by MMPs or other proteolytic enzymes. Both MMP2 and MMP9, among others, have been associated with hMSCs invasion capacity[38], while an increase in MMPs has been associated with IVD degeneration[39,40]. Herein, both MMP2 and MMP9 activities were evaluated. Although MMP2 and not MMP9 activity was detected, we could not distinguish between MMP production by hMSCs or bovine IVD cells. Hence, the mechanism by which cells migrate from the CEP to the IVD tissue is not fully elucidated. Future studies need to be designed to evaluate the contribution of MMPs and other enzymes in the migration process, in this particular model.

The effect of hMSCs seeded on CEP on the matrix of both NP and AF was then evaluated after 21 days in culture. hMSCs treatment through the CEP had a positive effect on proteoglycans, as suggested by the intense signal in the SO-FG staining in the IVDs of C + hMSCs treated discs, and an increased Agg expression in the NP, at both cellular and matrix level. Agg, the major proteoglycan constituent, has a key role in the maintenance of the osmotic properties of the IVD[41]. Agg cellular expression was quantified using ImmunoCellCount software, which allowed a faster and more accurate analysis of cell associated Agg deposition in the IVD tissue, thus encouraging the use of this software in future analysis to count Agg+ cells in IHC sections.

Previous studies have reported an upregulation of both Agg and Col type II gene expression in the presence of hMSCs, in *ex vivo*[30] and *in vivo* setups[42], for example in a porcine model[43]. Col type II is known to be present in both AF and NP, being more abundant in the NP area, while Col type I is the main component of the AF[1]. In this study, Col type II was also quantified and a significant in the NP of hMSCs-treated IVDs was observed, suggesting that the hMSCs were not only able to re-activate the synthesis pathways of Agg but also of Col type II. In addition, Col type I expression in the IVD tissue was analyzed. Col type I expression was more pronounced in the AF areas, decreasing towards the NP. Still, a higher expression of Col type I was observed in the NP of both lesion groups (cavity and C + hMSCs). In fact, the progression of the IVD degenerative process has been associated with alterations in collagen

types distribution, such as an increase in Col type I in the NP area[44], which is in accordance with the results observed in this model.

The ECM ultrastructure of bovine IVDs in culture was also explored. Dense ECM, namely collagenous fibrils and proteoglycans, and sparse AF/NP cells were observed, similarly to what was described for human IVD[45]. Higher ECM density was observed in both control and C + hMSCs groups with a more consistent fiber diameter being observed in control discs; while more heterogeneous fibers were observed in both cavity and C + hMSCs groups. Fiber heterogeneity present in the cavity and C + hMSCs groups might be due to tissue remodeling post-lesion. Smaller collagen fibrils suggests for the synthesis of new fibers and less uniform collagen fibers were previously described in human degenerated NP[46]. To further characterize these fibers, analysis and quantification of birefringent collagen fibers by the picrosirius-polarization method was performed[47,48]. This staining allows to distinguishes between mature and immature collagen fibers, although some authors also relate fiber polarization colours with collagen types[49]. Col type I is associated to the red/orange colours while Col type II presents variable colours depending on the tissue and species[50]. Higher percentage of red fibers was found in the NP of lesion groups (cavity and C + hMSCs), suggesting an increase in Col type I, corroborating our IHC findings. The highest ratio of greenish/reddish fibers was observed in the NP of control discs and a slightly higher amount of green fibers was observed in the hMSCs-treated group, comparing to the cavity, indicating an increase in the amount of immature fibers and thus suggesting *de novo* ECM synthesis. Still we cannot exclude the contribution of Col type II, significantly increased in the NP of C + hMSCs group, as this collagen type might also assume these polarization colors.

Behind the beneficial effect of MSCs on ECM of IVD could be their capacity of differentiation into IVD-like cells[43]. MSC differentiation towards NP-like cells has been described and associated with the production of insulin-like growth factor (IGF-1), basic fibroblast growth factor (FGF-2), platelet derived growth factor (PDGF)[51], and growth and differentiation factor-5 (GDF 5)[52]. Herein, we have investigated the associated growth factors in IVD culture media using an antibody array, post treatment of nucleotomized discs with hMSCs. This analysis revealed that 7 (out of 42) growth factors were more concentrated in C + hMSCs group, while only 1 was increased in the cavity group. The majority of the growth factors detected are known to be involved in ECM synthesis. For example, FGF-6 was shown to support MSC chondrogenesis, together with transforming growth factor-beta-2 (TGF-beta2)[53] and FGF-7 was suggested to induce chondrocyte proliferation[54]. A decrease in PDGFR alpha was detected in the C + hMSCs group: PDGF was shown to stimulate proteoglycan synthesis and cell proliferation in cartilage[55] and PDGFR is down-regulated or lost in cartilage-forming areas, as chondrogenic differentiation occurs[56], which could explain the results observed. In addition, IGF-1 receptor (IGF-1 sR), a key regulator of chondrogenesis

and proteoglycan metabolism[57], was also increased in the C + hMSCs group. IGFBP 6 is a factor involved in IGF transport in AC, highly abundant in bovine cartilage[58,59]; GM-CSF is a factor involved in Col type II and proteoglycan synthesis by rat chondrocytes[60] and PLGF is commonly associated with bone remodeling/regeneration and cartilage turnover[61]. Importantly, we exclude hMSC differentiation towards osteogenic lineage by Alizarin staining (see supplementary data). SCF, which was also augmented in the C + hMSCs group, has been described as a key stimulus in the regulation of proteins involved in hMSC proliferation and chondrogenesis[62]. Overall, all the growth factors identified correlate well with an augmented production of cartilaginous ECM. Still, we cannot exclude whether other growth factors might be highly expressed inside the IVD tissue, or if these growth factors were produced by hMSCs or by IVD cells stimulated by hMSCs. Nevertheless, our data suggests that hMSCs in CEP contribute to IVD ECM remodeling via a paracrine effect and that the beneficial presence of hMSCs relies on a combination of different growth factors, cytokines and other molecules. In the future more studies should be conducted to dissect which IVD cell subsets are specific targets of MSCs.

Although a clear increase of Agg and Col type II expression/synthesis in the NP was observed with our approach, we cannot exclude that ECM remodeling could be further enhanced by injection of growth factors such as bone morphogenic proteins (BMPs), transforming growth factor-β (TGF-β), growth/differentiation factor 5 (GDF-5) which could stimulate ECM production and cell proliferation[63], or even using short peptides such as LinkN, which have shown similar effects to MSCs injection in IVD ECM[64]. The use of techniques such as the "priming" of MSCs by cell transfection with non-viral vectors with GDF-5[65], or mechanical stimulus in bioreactors, which could provide specific mechanical or hydrostatic loading and hypoxia, could also benefit our strategy[66]. Besides, we cannot exclude that our results could be further enhanced by maintenance of IVD cultures under dynamic loading.

The CEP structure has a key role in IVD homeostasis by balancing the nutrition through its porous structure and by providing stiffness to resist axial loading[67]. Although it can represent an alternative route to degenerated IVD treatment by avoiding the injection through AF, this approach might be limited. For example, in patients where degeneration was initiated due to the blockage of nutrients diffusion in the CEPs, as in some trauma patients[66] or in patients with double-layer CEPs with increasing thickness[68], where the capacity of cells to migrate might be impaired. In those cases, other surgical approaches need to be developed to unblock the CEPs, such as the microfracture approach currently applied to stimulate articular regeneration[69]. To date, it remains unclear at which degeneration level should MSCs-based therapies be applied, but we believe that success would be increased if applied in early degenerative levels in an attempt to repair or delay further degeneration.

This work supports the concept of MSC migration to promote regeneration of degenerated IVD, as an alternative to cell transplantation, using the CEP approach as a main route for MSCs.

CONCLUSIONS

This work provides new insights on cell therapies and ECM remodeling in the IVD. The results obtained suggest that stem cell migration to IVD can improve local cell activity and ultimately tissue repair through the synthesis of matrix components. This reinforces the relevance of alternative strategies to MSC transplantation, such as the injection of molecules, which can trigger endogenous cell migration from local niches. Future studies will improve our understanding of the signaling pathways behind hMSCs effect in the IVD and reveal the relevance of MSC recruitment to IVD by chemoattractors for the spine field.

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CHAPTER IV

CHAPTER IV – Supplementary Data

SUPPLEMENTARY DATA

Mesenchymal Stem/Stromal Cells seeded on cartilaginous endplates promote Intervertebral Disc Regeneration through Extracellular Matrix Remodeling

Supplementary Data

hMSCs viability and survival in IVD culture media containing 2% of hMSCs tested serum

hMSCs were cultured in the normal medium for hMSC expansion (low-glucose Dulbecco's modified Eagle medium (DMEM, Gibco) and 1% Pen/Strep) containing 10% of MSC tested FBS. As a control for cell survival and viability, a preliminary experiment was performed using the IVD medium (DMEM 4.5 g/L glucose, 1% Pen/Strep, 1% ITS, 0.1% Primocin) which contains only 2% of FBS. hMSCs were cultured during 48h in these two media and afterwards, cell death and apoptosis were assessed by FACS using Ethidium and Annexin V FITC staining. No differences were observed in cell death and apoptosis levels when cultured with 10% FBS or 2%FBS (Figure 1S), giving indications that a low % of FBS in IVD culture would not compromise hMSCs viability in the IVD organ culture

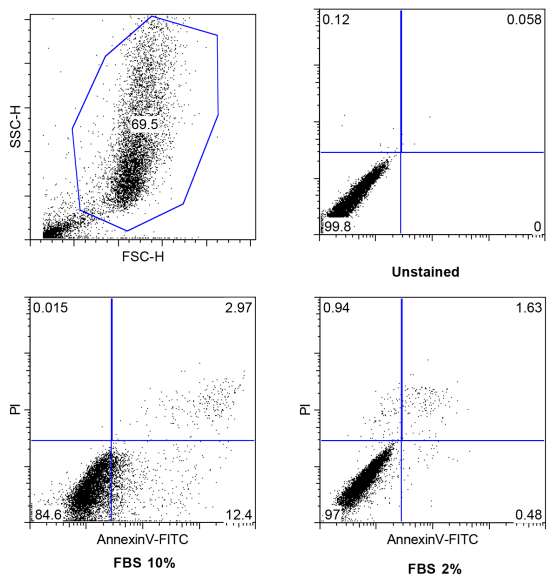

Figure 1S | hMSCs apoptosis analysis in MSC and IVD culture media.

Metalloproteinases Quantification

IVDs culture media was collected and analysed by zymography at different time points. Matrix metalloproteinases (MMPs), namely MMP-2 and MMP-9 activity was assessed by gelatin zymography as previously described (Cardoso, A.P. et al. Macrophages stimulate gastric and colorectal cancer invasion through EGFR Y(1086), c-Src, Erk1/2 and Akt phosphorylation and smallGTPase activity. Oncogene, 2014). Levels of pro-MMP2 and MMP2

were augmented in C+hMSCs group (Figure 2S). No pro-MMP and MMP9 were detected in the samples in the different time points.

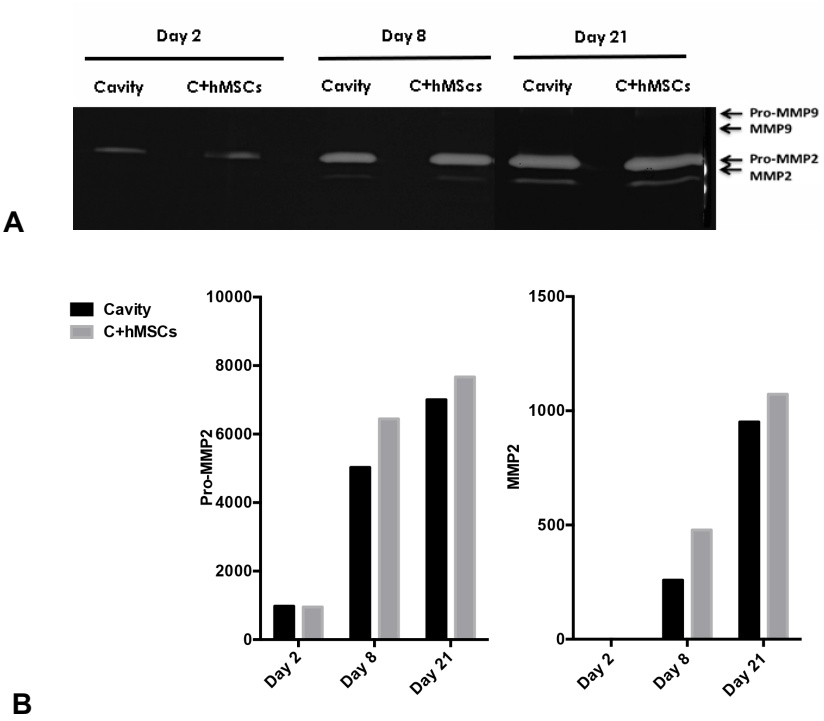

Figure 2S | MMP2 and MMP9 activity in IVD culture media overtime. **A |** Zymogram gel at different time points. The variants of MMPs are shown on the right. **B |** The intensity of gelatin digested bands by pro-MMP2, MMP2 were measured by densitometry and represented in the diagram bar.

Calcification in IVD tissue

The transplantation of MSCs into the IVD is sometimes associated with the presence of calcifications resulting from a differentiation towards a more osteogenic lineage over a disc-like/chondrogenic differentiation. To discard this hypothesis, we have performed a common staining for the identification of calcifications in the tissue, alizarin staining, on non-decalcified IVDs. Representative images of the staining can be observed in Figure 3S. A dot-deposition of calcium was observed in the AF of 2/3 of animals analyzed, unrelatedly with presence of hMSCs. No calcifications could be found in the NP area in any group. Therefore, it is our believe, that those calcifications were inherent to the isolated discs from each animal, and not a result of a hMSCs differentiation.

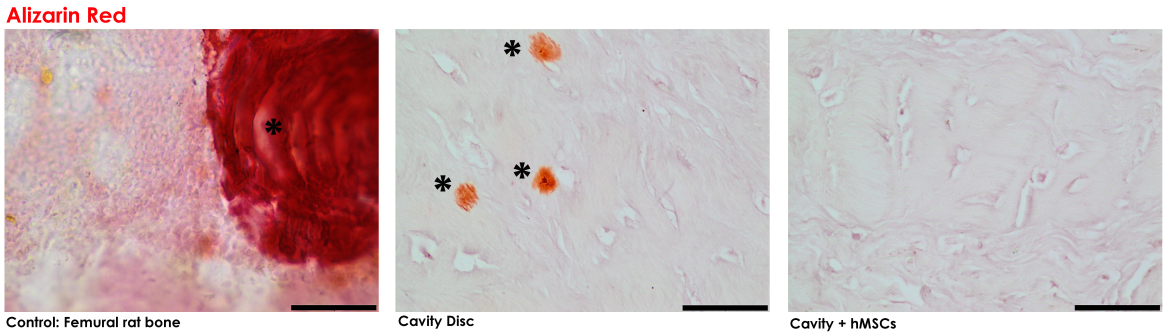

Figure 3S | Alizarin staining in IVD tissue. Alizarin (red*) staining in femural rat bone (positive control, left). Representative imagens of one animal in the AF area. Both in cavity discs (middle) and discs treated with hMSCs (right), calcification dots could be observed in the AF, but not in the NP. (Scale bar = 50 μm).

Aggrecan IHC quantification

IHC was performed in IVD section and aggrecan deposition was found to be co-localized with cells or in the very near neighborhood. Images were captured with an Olympus CX31 light microscope equipped with a DP-25 camera (Imaging Software CellˆB, Olympus, Center Valley, PA, USA) with the 20x objective to have a compromise between a statistically relevant number of cells and a proper color representation of the cells (provided by a sufficient number of pixels associated with every cell). IHC positive cells (Agg+) assume a brownish color while negative cells are purplish, allowing distinction between the two classes. The total number of cells and the number of Agg+ cells were determined either manually or using the ImmunoCellCount Software.

Software design

The ImmunoCellCount software was implemented in MATLAB with the objective of developing an approach for unbiased, consistent, automatic and faster counting of Agg+ cells in IHC images of IVDs. This software provides a simple and user-friendly graphical user interface (GUI), and works in two stages: the first is responsible for cell identification (segmentation), and the second is responsible for cell classification. Two parameters are required for cell identification: an intensity threshold (for the segmentation algorithm) and a cell size (for object enhancement through a Laplacian of Gaussian filter). Cell classification is performed by measuring distances in color space: the user sets the reference colors for a negative cell (purple) and a positive Agg+ cell (brown); all cells found in the first stage are classified according to which reference color they are closer to (measured in RGB color space).

All parameters, including reference colors, only need to be set once for a set of images sharing the same acquisition conditions. This method provides an unbiased and consistent procedure to classify and count positive and negative staining. To validate ImmunoCellCount, 12 images (more than 500 cells) were analyzed manually (detailed visual inspection of color) and with the software.

Collagen II quantification.

IF was performed in IVD section and collagen expression intensity was quantified in the images in both AF and NP areas of the disc. Images were captured using an inverted microscope, Axiovert 200 M, Zeiss with the 10x objective. The intensity of collagen expression in the tissue was determined using an additional custom made MATLAB script, the IntensityStatisticsMask Software.

Software design

The purpose behind the construction of this script was also to obtain a faster and unbiased method, now for color intensity quantification. The script performs image segmentation, based on a user-defined threshold level, to create a mask for the tissue regions expressing collagen. Intensity measurements, such as mean values and standard deviation, are then calculated only for the pixels belonging to the collagen mask. Unless otherwise stated, the intensity threshold value used for the segmentation was 10.

Transmission Electron Microscopy

The ultrastructure characterization of the IVD by transmission electron microscopy (TEM) allows a more profound visualization of matrix/cell alterations that occur during the degenerative process. At cellular level, both active and healthy cells and necrotic cells could be observed in all the groups, although non-representative numbers of cells were observed. Healthy cells had signs of cell activity and matrix synthesis (normal cell nucleolus, intact cytoplasm and organelles; dense ECM matrix components encircling cells) (Figure 4S A), while necrotic cells presented signals of chromatin clumping and, in some cases, cytoplasmatic deterioration (Figure 4S B).

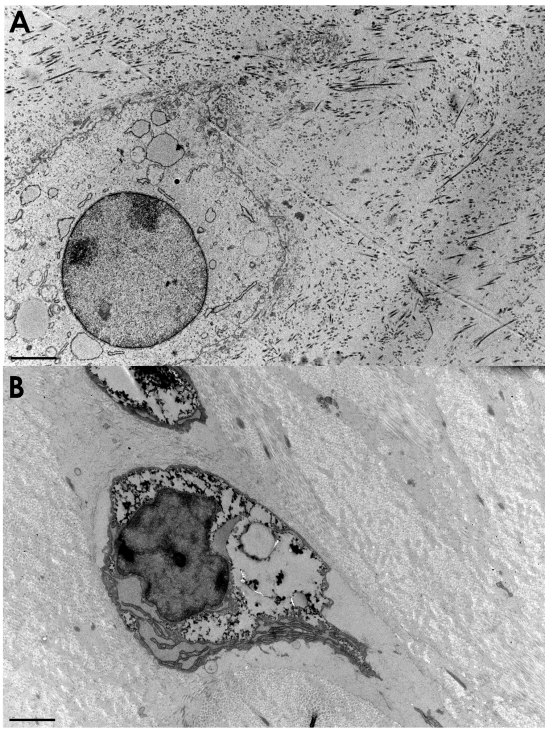

Figure 4S | IVD cells observed by TEM. A: healthy cell. B: necrotic cell**.**

Growth Factors Analysis

A commercially available array of growth factor proteins (RayBio® C-Series Human Growth Factor Antibody Array C1, #AAH-GF-1-4, RayBiotech, Inc. 3607 Parkway Lane, Suite) was used to evaluate the relative levels of growth factors production in the IVD culture media of both Cavity and C+hMSCs at day 21. The array is composed of 42 GFs, as depicted in Figure 5S. A pool of 6 donors from the Cavity and C+hMSCs was analyzed. Results were generated by quantifying the mean spot pixel density from the array using image software analyses (ImageLab 4.1; BioRad). Briefly, the pixel intensities gathered from the array spots were obtained using the volume tools option of the software. We have defined an area of interest of the reference spots by surrounding it with a circle, and then equal circles were used for all spots of the array. The densities of signals obtained were normalized with the background. Results were afterwards represented as a color gradient of ranges of growth factors levels released in the IVD culture media. Each color representing a range of concentrations as indicated on the right table. For the results interpretation, we considered alterations of a fold change >2 or <0.5.

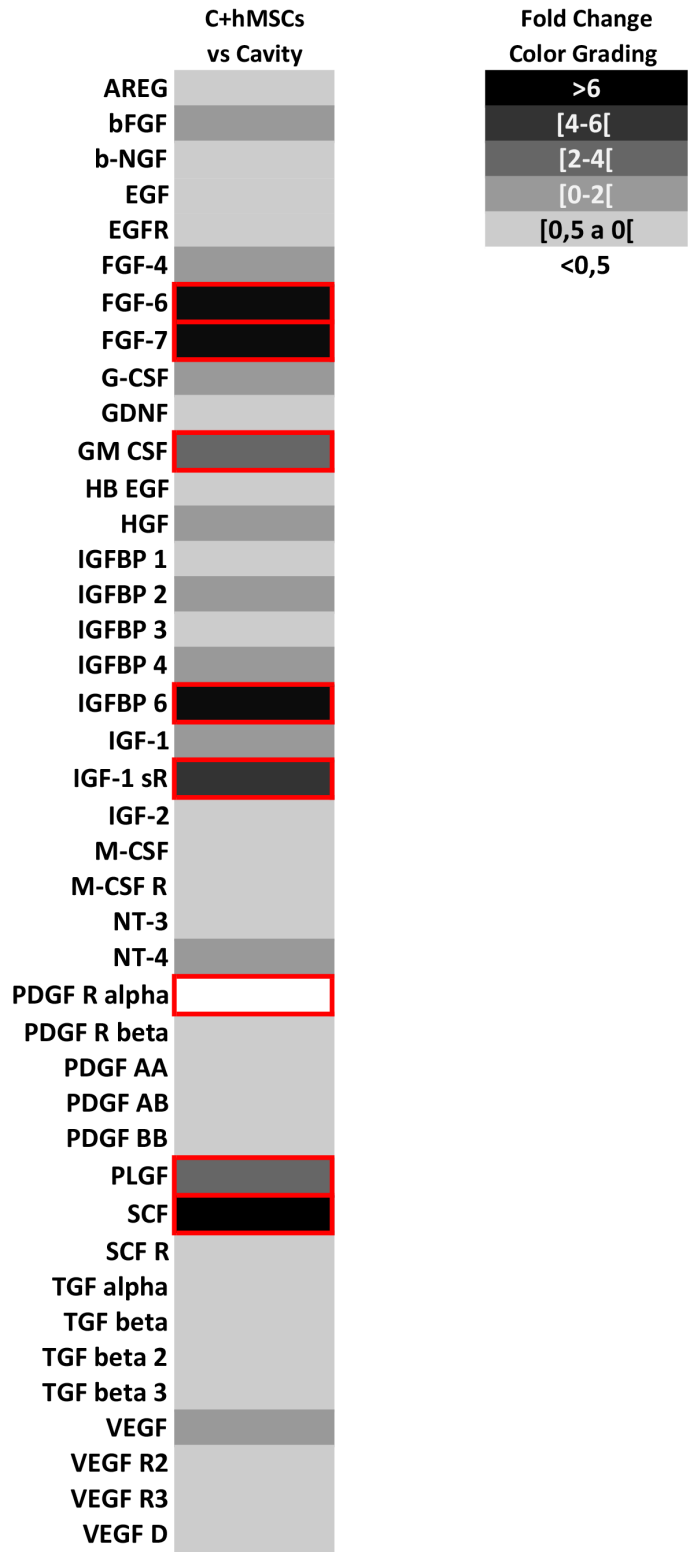

Figure 5S | Color gradient representation of ranges

CHAPTER V

CHAPTER V - ARTICLE 2

THE EFFECT OF HYALURONAN-BASED DELIVERY OF STROMAL CELL-DERIVED FACTOR-1 ON THE RECRUITMENT OF MSCS IN DEGENERATING INTERVERTEBRAL DISCS

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The effect of hyaluronan-based delivery of stromal cell-derived factor-1 on the recruitment of MSCs in degenerating intervertebral discs.

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ABSTRACT

Intervertebral disc (IVD) degeneration is the leading cause of low back pain and disability in the active population. Transplantation of mesenchymal stem cells (MSCs) in a hydrogel carrier can induce regenerative effects in degenerated IVDs. Moreover, it was found that degenerative discs release chemoattractants effective in MSC recruitment. Based on these findings, we hypothesized that an injectable hydrogel that can enhance the number of migrated MSCs in the IVD and provide a suitable matrix for their survival and differentiation would be ideal. The purpose of this study was to evaluate the potential of a thermoreversible hyaluronan-poly(N-isopropylacrylamide) (HAP) hydrogel as chemoattractant delivery system to recruit human MSCs in degenerative IVDs. The results demonstrate that HAP hydrogels containing stromal cell derived factor-1 (SDF-1) significantly increased the number of MSCs migrating into nucleotomized discs compared with discs treated with only HAP or SDF-1 in solution. HAP hydrogels releasing SDF-1 enhanced both the number of recruited cells and their migration distance in the IVD tissue. Furthermore, this phenomenon was dependent on MSC donor age. In conclusion, HAP SDF-1 is effective for the recruitment of stem cells in the IVD, thus opening new possibilities for the development of regenerative therapies based on endogenous cell migration**.**

Keywords: Mesenchymal stem cells; Recruitment; Thermoreversible hyaluronan hydrogel; Stromal cell derived factor-1

INTRODUCTION

Low back pain (LBP) is recognized as one of the leading causes of absenteeism from work and severe life quality deterioration. This burden has been estimated to affect more than 70% of the population at least once in their lives and may be caused by various factors[1]. Intervertebral disc (IVD) degeneration is now accepted as the major pathophysiological mechanism responsible for LBP[2]. The IVD degenerative process, contrarily to other musculoskeletal tissues, has an early onset and can already be identified in adolescence[3,4]. Biologically, the hallmark of IVD degeneration is an altered cell metabolism due to de-regulations in cellular phenotype. These changes result in an unbalanced synthesis of matrix components, accumulation of free radical species, decrease of pH and aberrant activity of proteolytic enzymes[5]. As a consequence, histomorphological modifications are observed in the disc, and the whole spine biomechanics becomes severely affected[6].

Current treatments range from conservative approaches such as physical therapy and analgesic drugs to invasive procedures like discectomy or spinal fusion. The latter can result in a significant loss of flexibility and increased risk for adjacent disc degeneration[7]. Fundamentally, none of the therapeutic options available restore the normal biological function of the spine[6]. Therefore, it is critical to develop new therapies for early IVD degeneration. In the past years, several biomaterial-based therapies have been proposed for IVD replacement or nucleus pulposus (NP) substitution. The total replacement of the disc by prosthetic devices has shown limited efficacy and critical disadvantages such as device migration and the need for revision. Moreover, prosthetic devices, either metallic or polymeric, have shown inability to remodel and sustain the loads on the spine[6]. Other materials, such as hydrogels, have emerged as alternatives for NP substitutes. Hydrogels resemble the native extracellular matrix (ECM) structure and mechanical properties, may be functionalized with target molecules to stimulate regeneration, or can be loaded with cells[8].

Still, the extrusion of the materials through the annulus fibrosus (AF) constitutes a major problem[9]. Other approaches include the use of cellular-based therapies. Within cell-based therapies, human mesenchymal stem cells (hMSCs) hold great promise mainly because of their self-renewal capacity and multilineage differentiation potential, which make them the perfect candidate for regeneration of various tissues[10]. hMSCs have been shown to be able to differentiate into NP-like cells, expressing markers as SOX-9, col type II and agg, and more specific IVD markers such as FOXF1 or CA12[11-13]. The direct injection of hMSCs in the IVD has been tested in human pilot trials; however, while some pain relief was reported, no effective disc regeneration was observed[14,15]. In fact, cell transplantation to sites of lesion has been associated with low cell viability postinjection, although cell carriers, such as hydrogels, may overcome this drawback[16]. Certain hydrogels have shown promising results, promoting

both the viability of injected cells as well as their differentiation into NP-like cells[17,18]. Moreover, transplantation of MSCs in a hydrogel carrier induced regenerative effects in degenerated IVDs[17]. Nevertheless, the long-term survival of the implanted cells remains unknown and doubtful, particularly taking into account the limited diffusion of nutrients to the IVD niche.

hMSCs also have the ability to migrate to injured tissues and interact with the surrounding environment through the secretion of a broad range of molecules like growth factors, cytokines and chemokines[19,20]. Some of the molecules that promote hMSC recruitment are CXCL12/SDF-1 (stromal cell derived factor-1), RANTES (regulated on activation, normal T-cell expressed and secreted) or MDC (macrophage-derived protein)[21]. Recently, it was demonstrated in an *ex vivo* model that hMSCs can be recruited by the degenerative IVD environment[22]. Moreover, it was found that degenerative discs release chemoattractants effective in MSC recruitment[23]. Nonetheless, the percentage of migrated cells to injury sites might be limited due to the cellular signals that control the cell trafficking[24].

hMSC mobilization might be improved by the local increase of cytokines/chemokines released using appropriate carriers. For example, SDF-1 is a well-known chemokine, constitutively produced in the bone marrow and strongly chemotactic to lymphocytes, monocytes, endothelial progenitor cells[25,26] and hematopoietic stem cells. SDF-1 has also been shown to recruit hMSCs[27]. It has been demonstrated that SDF-1 incorporation in proper delivery systems is able to promote cell recruitment to an injury site and increase the potential of tissue regeneration: Shen et al. were able to improve tendon regeneration by the implantation of an SDF-1 releasing silk-collagen scaffold[28]; SDF-1 incorporation in alginate, either as a gel or scaffold, was used for regeneration of osteochondral defects[29] and skin in a wound healing scenario[30]. Nevertheless, these carries lack critical properties for an application in the IVD, such as injectability, for a less invasive procedure, as well as the ability of gelation in situ. Therefore, SDF-1 release in the disc using an appropriate delivery system might represent an increase of IVD regenerative potential.

The thermoreversible hyaluronan-poly(N-isopropylacrylamide) (HAP) hydrogel, composed of hyaluronan (HA), a key ECM component, and poly(N-isopropylacrylamide) originated a thermoreversible and injectable co-polymer that has the ability to rapidly gellify in situ (>30ºC)[31,32]. These properties turn HAP into an attractive candidate for applications in the IVD. Moreover, HAP is cytocompatible and has been shown to support hMSC differentiation towards NP-like cells by the up-regulation of collagen II, SOX-9 and also KRT19, CD24, and FOXF1 expression, recently described as IVD markers[31,33].

Based on these findings, we hypothesized that HAP hydrogel may serve both to enhance the number of migrated MSCs in the IVD and to provide a suitable matrix for their survival and differentiation. The purpose of this study was to evaluate the potential of HAP hydrogel as a chemoattractant delivery system to recruit hMSCs in degenerative IVDs. With

that aim we incorporated SDF-1 into HAP hydrogel and assessed: 1) SDF-1 release kinetics; 2) hMSC migration in an *ex vivo* model of degenerating IVD; and 3) the impact of hMSC donor age on cell migration. If successful, this strategy could open new perspectives on cell-based therapies for IVD regeneration.

MATERIALS AND METHODS

Intervertebral disc harvest

IVDs with cartilaginous endplates (CEP) were isolated from young (5–8 months old) bovine tails (*n* = 10) obtained from the local abattoir (Davos, Switzerland). Six caudal discs from each bovine tail were harvested following a protocol previously described[34]. Briefly, the caudal discs with CEP were removed using a band saw (Exakt Apparatebau GmbH, Germany) to obtain parallel cuts. The CEPs were afterward jet-lavaged with Ringer balanced salt solution (Braun, Germany) using a Pulsavac wound debridement irrigation system (Zimmer, Inc., Switzerland). Discs were washed sequentially in 1%/10%/1% of Penicillin/Streptomycin (Pen/Strep) in a phosphate-buffered saline solution (PBS) (pH = 7.4) for 1/10/1 min, respectively. Afterward, discs were incubated overnight in 6-well plates with high-glucose Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 2% fetal bovine serum (FBS, Gibco), 1% Pen/Strep (Gibco), 1% insulin–transferrin–selenium supplement (ITS, BD, Becton Dickinson) and 0.1% Primocin (Invivogen) at 37 °C in a 5% CO_2 atmosphere.

Isolation and culture of hMSCs

Human bone marrow aspirates were obtained from the University Hospital of Bern after approval by the local ethical commission (KEK188_10) and written consent of the patient. hMSCs were isolated by Ficoll® gradient centrifugation and adherence to tissue culture plastic as previously described[22]. Cells were expanded in alpha-minimum essential medium (α-MEM, Gibco) containing 10% FBS and 1% Pen/Strep for expansion and used at passages P3–P4 for the recruitment studies. A total of 5 human donors were used (Table 1).

Table 1 | Information of Human Mesenchymal Stem Cells (hMSCs) donors.

DONOR	AGE	GENDER
#h1	19	Female
#h2	21	Female
#h3	24	Male
#h4	40	Female
#h5	64	Female

HA-based delivery system for SDF-1

HA Sodium salt from streptococcus equi was purchased from Contipro Biotech s.r.o. (Czech Republic) with a weight-average molecular weight M_w = 1.5 MDa and polydispersion index = 1.53. Amino-terminated poly (N-isopropylacrylamide) (pNIPAM-NH_2) of 44 ± 2.7 kDa was purchased from Polymer Source, Inc (Canada). HAP was prepared by direct amidation

reaction of thermoreversible segments of pNIPAM-NH2 on HA backbone, as previously described by D'Este et al.[35].

The polymer solution was filter-sterilized, lyophilized and then reconstituted in PBS (pH 7.4) at a concentration of 10% wt/vol, containing different amounts of the human chemokine SDF-1 (Peprotech, UK). The release of SDF-1 from HAP *in vitro* was assessed following a method described elsewhere[36]. Briefly, 50 µL of HAP hydrogels carrying 3 ng/µL of SDF-1 were cast into 2 mL tubes and gellified in a thermo mixer (37 °C) for 10 min. Afterward, 1.5 mL of pre-warmed medium (serum-free DMEM) was added to the tube and incubated at 37 °C with shaking (225 rpm) during 7 days. At pre-determined time points – 0, 2, 4, 6, 24, 48 and 168 h – half of the medium (0.750 mL) was collected and replaced by the same volume of fresh medium. The harvested medium was stored for further analysis.

For the *ex vivo* IVD culture, which will be described in more detail in the following section, two SDF-1 concentrations were prepared to achieve maximum theoretical releases of 100 and 200 ng/mL (Table 2), based on previous studies[27]. The gel containing the different formulations was injected in an IVD defect, and the release of SDF-1 was measured in the culture medium at pre-determined time points (0, 24 and 48 h). The release of SDF-1 in both systems was assessed by quantifying the chemokine in the harvested medium using an Enzyme-Linked Immunosorbent Assay (ELISA) (Human CXCL12/SDF-1 ELISA Development kit, R&D Systems, USA).

Table 2 | Formulations of Hyaluronan-poly(*N*-isopropylacrylamide) (HA-pNIPAM) with Stromal Cell Derived Factor-1 (SDF-1) for injection in the *ex vivo* model.

Formulation	HAP SDF 5	HAP SDF 10
Volume of Gel injected (µL	50	50
SDF-1 concentration in the Gel (ng/µL)	5	10
Volume of Medium (mL)	2.5	2.5
Maximum SDF-1 Concentration (ng/mL)	100	200

Ex vivo IVD organ culture model

The IVDs prepared as described were randomized for the following 6 study groups:

1. Intact disc (Intact);
2. Discs with an empty cavity (Cavity);
3. Discs with a cavity filled with HAP gel only (HAP);
4. Discs with a cavity filled with HAP with 5 ng/µL of SDF-1 (HAP SDF 5) (Table 2);
5. Discs with a cavity filled with HAP with 10 ng/µL of SDF-1 (HAP SDF 10) (Table 2);
6. Discs with a cavity filled with 5 ng/µL of SDF-1 solution in PBS (SDF 5).

For groups 2–6, a previously developed model of IVD access through the CEP was adopted for chemoattractant delivery[32]. Briefly, a circular cavity was made in the CEP using a 4 mm biopsy punch. The portion of the CEP was removed and kept in PBS with 1% Pen/Strep, while part of the NP (0.05–0.1 cm^3 of tissue) was removed using a blade. This cavity was then filled with different formulations of the gel or remained empty. In the groups containing HAP, the discs were incubated at 37 °C immediately before gel injection to allow fast gelation of the hydrogel. Then, the removed CEP was repositioned, sealed with bone cement (PMMA, Vertecem V Cement Kit, Synthes, Switzerland) and incubated for 30 min, allowing the cement to dry. Finally, all the discs were turned to place the cavity at the bottom of the well and incubated in medium (DMEM 4.5 g/L glucose, 2% FBS, 1% Pen/Strep, 1% ITS and 0.1% Primocin) in 6-well plates for 2 h.

Cell recruitment assay

hMSCs (P3–P4) were labeled with PKH26 (red) following the manufacturer instructions (PKH Fluorescent Cell Linker kit, Sigma–Aldrich, Germany). Immediately after labeling, PKH26-hMSCs were seeded (1 × 10^6 cells/disc) on the top of the CEP (i.e. on the side opposite to the injury) and incubated for 30 min to allow cells to adhere to the CEP structure. Afterward medium (DMEM 4.5 g/L glucose, 2% FBS, 1% Pen/Strep, 1% ITS, 0.1% Primocin) was added. Discs were cultured at 37 °C in a 5% CO_2 atmosphere incubator, and cell migration was allowed to occur during 48 h (Figure 1). After this period, discs were extensively washed with PBS, briefly incubated with trypsin to remove cells that remained on the surface and fixed in 4% buffered formalin for 3–4 days. This procedure was repeated for each individual donor of hMSCs (n = 5), using two bovine tails per donor (n = 10 experiments).

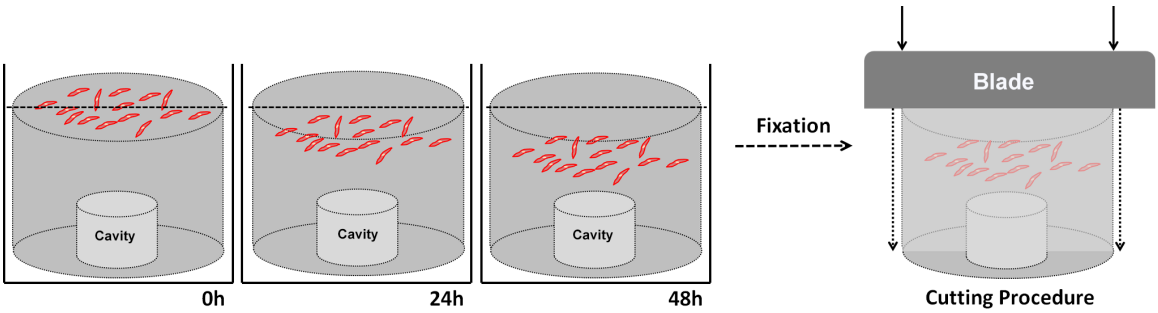

Figure 1 | Schematic representation of the experimental design used for cell migration experiments. *Ex vivo* intervertebral disc (IVD) organ culture model was developed using a cartilaginous endplate (CEP) approach to create a cavity in the nucleus pulposus (NP). PKH26 (red)-labeled hMSCs were seeded on the top of the CEP (opposite to the cavity) and allowed to migrate during 48 h. After this period, discs were fixed and cut sagittally.

Cell recruitment analysis

After fixation, discs were cut sagittally (Figure 1), using a custom-made guillotine system with a Dermatome Blade (Integra Life Sciences Corporation, USA). The two sagittal sections obtained were analyzed separately on a motorized Axiovert 200M microscope (Zeiss, Germany) at 2.5× magnification. In the first channel, the 488-nm wave length of the Argon laser was used to distinguish the disc structure with green autofluorescence; in the second channel, the 543-nm wave length of HeNe1 laser was used to image the red fluorochromes of the labeled hMSCs, as previously described[37]. Images were acquired using Mosaic software add-on (Zeiss). hMSCs were counted manually using AxioVision Software 4.8, following specific criteria (Figure 2):

1. The CEP where hMSCs were seeded was excluded from the counting;
2. The IVD sections were divided in 1 mm-height sub-sections (S1, S2, S3, S4, S5, S6 and S7) to facilitate cell counting;
3. The number of migrated cells per disc is the sum of the number of migrated cells in the two sagittal sections of the disc;
4. Only labeled hMSCs (10–80 μm size range) were considered for counting;
5. The NP and AF regions were discriminated based on disc autofluorescence (488 nm).

hMSCs were further detected in the bovine tissue by immunohistochemistry. For human nuclei detection, masked epitopes were exposed by treatment with 10 mm sodium citrate (pH 6) for 35 min at 95–98 °C. Paraffin sections were incubated overnight (4 °C) with mouse anti-human nuclei primary antibody (MAB4383–3E1.3 Millipore, 1:400); afterward, Alexa Fluor 594-labeled goat anti-mouse (Invitrogen-Molecular Probes, 1:1000) was used as the secondary antibody (1 h at room temperature). All sections were mounted in Fluorshield with DAPI (Sigma). Control sections for each immunolabeling excluded primary antibody staining. Representative images of the slides were taken using confocal laser scanning microscopy (CLSM) (Leica SP2, objective, HC PL APO CS 40×).

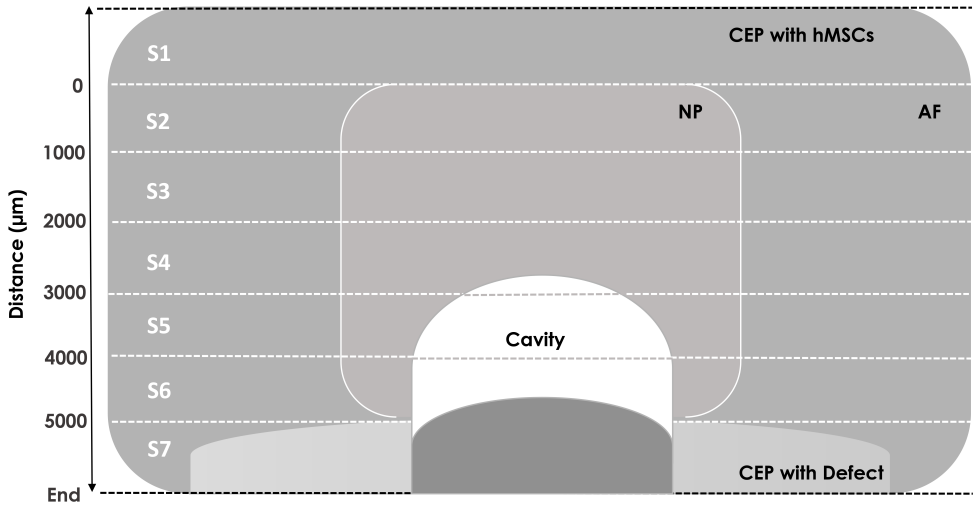

Figure 2 | Schematic view of the analysis setup to for cell migration assessment. Migrated cells were counted in the entire disc area, excluding the endplate ("CEP with hMSCs") where cells were initially seeded. The disc images were sectioned into 1 mm-height sub-sections (S) in order to assess cell migration in more detail: the line corresponding to "0 µm", limits the endplate border. The sub-section S1 corresponds to the area surrounding the CEP with hMSCs. The subsequent lines represent the sub-sections at distances ranging from 1000 µm to >5000 µm. The area of nucleus pulposus (NP) was also traced based on disc auto-fluorescence, which allows discrimination of NP and annulus fibrosus (AF).

Statistical analysis

Statistical analysis was performed using Prism 5.0a for Mac OS.X. The parametric distribution of the data was evaluated by D'Agostino and Pearson normality test. If data followed a normal distribution, statistical analysis was performed using the repeated measures ANOVA test followed by Bonferroni's comparison test for selected groups (e.g. percentage of migrated hMSCs). In the cases of non-parametric data, the Mann–Whitney test was used to compare two groups of non-related samples (e.g. younger *vs* older donors) and the Friedman test followed by Dunn's Multiple Comparison test was used to compare multiple related samples (e.g. hMSC migration of younger donors). Statistical significance was considered at least for $p < 0.05$ (*) (**: $p < 0.025$, ***: $p < 0.001$).

RESULTS

SDF-1 release from HA-based delivery system

The goal of this study was to incorporate SDF-1 into HAP hydrogels with the aim of recruiting hMSCs towards the IVD. SDF-1 incorporation did not affect the gelation temperature of HAP that occurs above 30 °C [31]. The HAP-SDF-1 hydrogel was incubated in serum-free culture medium (pH 7.4) at 37 °C and SDF-1 release kinetics analyzed by ELISA during 7 days (Figure 3). A burst release of SDF-1 (~27%) was observed within the first 6 h, after which the release continued slowly, reaching about 54% after 168 h. During this period, the gel showed no apparent visual signs of degradation.

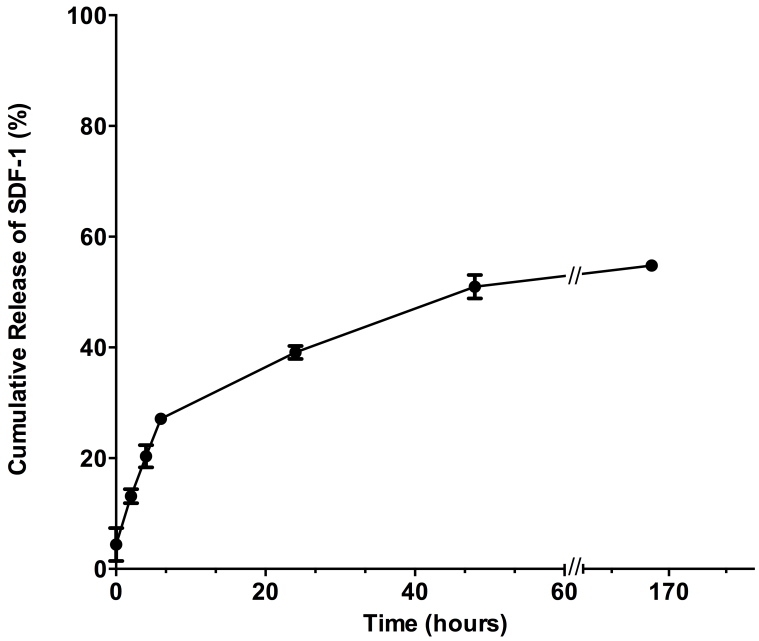

Figure 3 | *In vitro* stromal cell derived factor-1 (SDF-1) release kinetics from hyaluronan-poly(N-isopropylacrylamide) (HAP). HAP hydrogel with SDF-1 was cast into 2 mL tube and kept under stirring in serum-free DMEM at 37 °C for 168 h, simulating the standard cell culture conditions. The amount of SDF-1 released in the medium was assessed by ELISA. A burst SDF-1 release (~27%) was observed in the first 6 h, followed by a continuous increase, up to 54% after 168 h. Results are presented as Mean ± StDev (*n* = 3).

hMSC recruitment towards SDF-1-HA-based delivery system in an ex vivo IVD organ culture model

The feasibility of hMSCs to be recruited towards a degenerating IVD was first reported by Illien-Jünger et al.[22]. Herein we attempt to demonstrate that a chemoattractant-delivery system can enhance hMSC recruitment to IVD. Six study groups were defined as described earlier: Intact, Cavity, HAP, HAP SDF 5, HAP SDF 10 and SDF 5.

After 48 h, hMSC migration was analyzed by detecting the presence of PKH-labeled hMSCs in the IVD tissue by microscopy (Figure 4A and B). The presence of human cells in the IVD was further confirmed by labeling human nuclei by immunohistochemistry (Figure 4C). hMSC counts were expressed in percentage of migrated cells for each individual donor, in which 100% of cell migration corresponds to the maximum number of migrated cells detected for a single human donor in one disc (independent of the condition). The mean percentage of cell migration for all the donors tested (n = 5) is represented in Figure 5A.

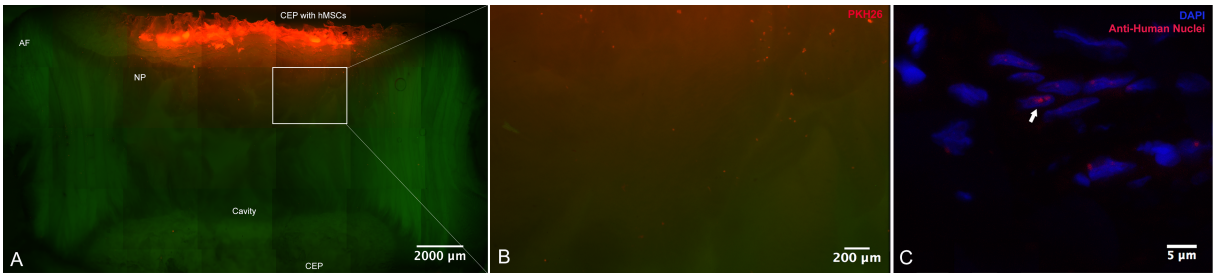

Figure 4 | Identification of hMSCs in the IVD tissue. A) Sagittal section of the IVD. hMSCs were counted manually using AxioVision Software 4.8. The IVD tissue autofluorescence, in green, allowed identifying the different areas of the disc: annulus fibrosus (AF); nucleus pulposus (NP) and cartilaginous endplate (CEP). PKH26-labeled cells in red can be observed in CEP where the cells were initially seeded and distributed along the AF and NP tissue towards the cavity. **B) Magnification (5×) of the sagittal section for cell counting**. Individual migrating PKH26-labeled cells can be observed and counted. **C) Immunohistochemistry of hMSCs in the bovine tissue**. Representative image of cells of both bovine and human origin were identified using DAPI (blue) and human cells distinguished using anti-human nuclei antibody (red, arrows). Images were acquired using CLSM (Leica SP2, objective, HC PL APO CS 40×, digital magnification 6×).

The results show that the percentage of migrated hMSCs slightly increased from Intact to the Cavity IVD (from 19 ± 25% to 34 ± 24%), although with a high variability between the different donors. In the presence of the HAP gel only, the percentage of hMSC migration slightly increased (to 48 ± 32%). However, when SDF-1 was incorporated into HAP gel, the

percentage of hMSC migration significantly increased in comparison with both Intact and Cavity controls (***, $p < 0.001$), and with the HAP group (*, $p < 0.05$), reaching 75 ± 26% and 81 ± 21%, for HAP SDF 5 and HAP SDF 10, respectively. No significant differences were found between the two concentrations of SDF-1 in the gel (5 and 10 ng/μL). When soluble SDF-1 was injected in the cavity, a slight increase in hMSC migration was observed (40 ± 26%), when compared with Intact and Cavity control groups.

In addition, to eliminate the possibility of cells being dragged by the blade during the cutting procedure, a control experiment was performed in which hMSCs were counted 30 min after cell seeding (data not shown). No cells were found in the AF or NP regions, which confirmed that hMSCs counted in the recruitment assays were effectively able to migrate in the IVD tissue.

We also discriminated the proportion of migrated cells in NP versus AF (Figure 5B). The results show that the majority of the hMSCs (~60%) were found in the NP, while approximately 40% were present in the AF. The spatial distribution of hMSCs was similar for all the groups, suggesting that hMSCs have the capacity to migrate through the ECM of both NP and AF.

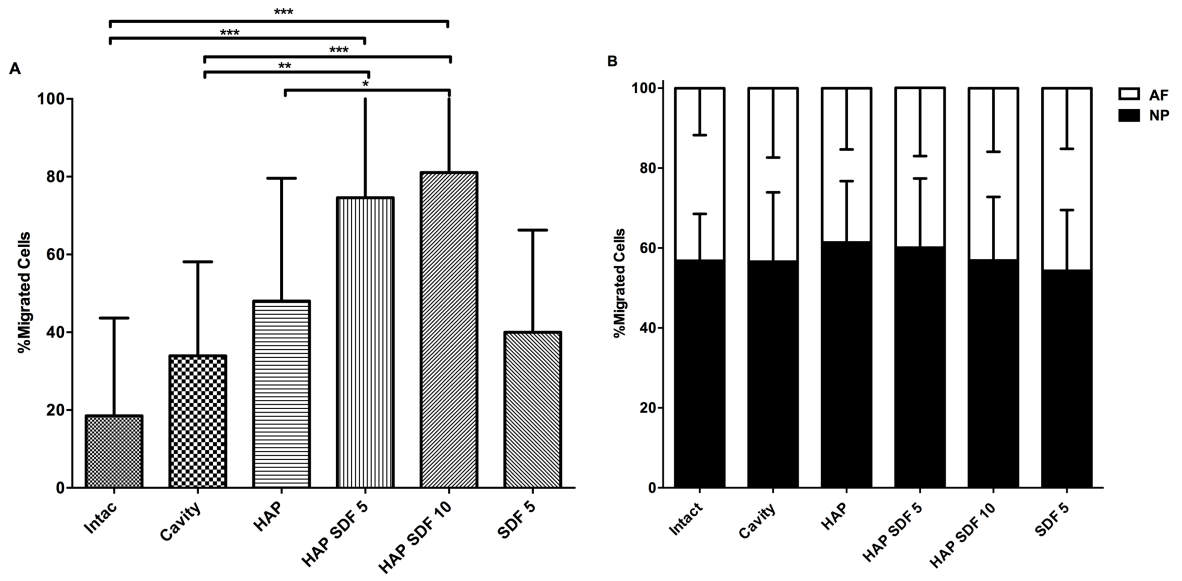

Figure 5 | Percentage of hMSC migration in the *ex vivo* IVD cultures. The average percentage of cell migration was obtained by normalizing the data for each donor to its maximum value of migration (100%). Percentage of migrated hMSCs is given for the study groups: Intact, Cavity, HAP, HAP SDF 5, HAP SDF 10, and SDF 5. A) Comparison of percentage of hMSCs migration in whole disc. A significant enhancement of hMSCs recruitment was only observed in the presence of HA-pNIPAM hydrogels with SDF-1. Results are presented as Mean ± StDev (n = 10), statistical significance was considered for p value (*) <0.05 (Repeated measures ANOVA, Bonferroni's multiple test *, $p < 0.05$; **, $p < 0.025$; ***, $p < 0.001$).B) Comparison of percentage of hMSCs migration in nucleus pulposus (NP) *vs* annulus fibrosus (AF). No significant differences were observed between the different study groups. Approximately 60% of the cells were present in the NP, while 40% were identified in the AF, suggesting that hMSCs are able to migrate through the matrix in both tissues. Results are presented as Mean ± StDev (n = 10).

SDF-1 release from HA-based delivery system in an ex vivo IVD organ culture model

SDF-1 release from HA-based delivery systems in the *ex vivo* model was analyzed by ELISA (Figure 6). SDF-1 concentration was residual in the organ culture medium (0–0.08 ng/mL) remaining in this range of values in Intact group (0.03 ± 0.07 ng/mL) during 48 h. In the Cavity and HAP groups the concentration of SDF-1 slightly increased, reaching 0.4 ± 0.6 ng/mL in both conditions after 48 h. This increase suggests that the disc cells may release SDF-1 in response to injury (the cavity). In the SDF-1-containing gels its concentration in the medium significantly increased (**, $p < 0.025$; ***, $p < 0.001$), reaching levels of 1.04 ± 0.6 ng/mL (HAP SDF 5) and 2.06 ± 1.6 ng/mL (HAP SDF 10) at 24 h. In the SDF 5 group, the highest mean concentration was obtained at 48 h (0.47 ± 0.7 ng/mL). These results show that SDF-1 incorporated in the HA-pNIPAM can be sustainably released in the *ex vivo* IVD organ culture, although the amounts detected were lower compared to the theoretical maximum amounts that could be released (100 and 200 ng/mL for HAP SDF 5 and HAP SDF 10, respectively) (Table 2).

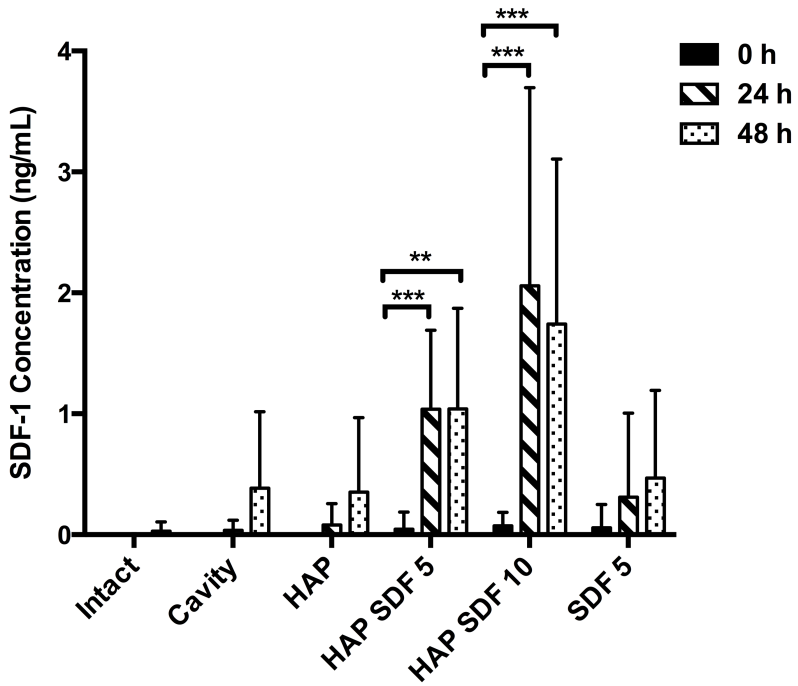

Figure 6 | Concentration of stromal cell derived factor-1 (SDF-1) in media of *ex vivo* IVD cultures. The concentration of SDF-1 in the medium of the *ex vivo* IVD culture was analyzed by ELISA during 48 h. Results are presented as Mean ± StDev ($n = 10$). Statistical significance was considered for p value (*) <0.05 *, $p < 0.05$; **, $p < 0.025$; ***, $p < 0.001$ (Mann–Whitney test).

Effect of hMSC donor age in cell recruitment in the ex vivo IVD organ culture mode

The age of hMSC donors is known to correlate with certain functional properties, such as their differentiation capacity[38]. Herein, the effect of donor age in hMSC migration was addressed. The number of migrated hMSCs from older (#h4 and #h5 – 40 and 64 years old) and younger (#h1, #h2 and #h3 – 19–24 years old) donors in the IVDs was compared (Figure 7).

After 48 h, increased numbers of hMSCs from younger donors were migrated into the IVD compared to hMSCs from older donors (Figure 7A). An increase in cell migration from younger *vs* older donors was observed in all the groups tested, ranging from 2.4 ± 0.9 fold (Intact group) to 3.8 ± 1.4 fold (HAP SDF 5). This increase was statistically significant (*$p < 0.05$) for all the groups tested, except for the Intact group (Figure 7A). This finding suggests that hMSCs isolated from younger donors are more prone to migrate when compared to older donors, both in the case of an injury (the Cavity and all the HAP groups) and in response to SDF-1.

When considering each age group separately, it can be observed that hMSC migration from older donors is not significantly different between the groups studied (Figure 7B), varying between 133 ± 110 to 233 ± 17 migrated cells per disc, although a slight increase was noticed in the groups with HAP SDF 5 and HAP SDF 10 (195 ± 28 and 233 ± 17, respectively). With hMSCs from younger donors, cell migration was significantly higher (*, $p < 0.05$) in discs injected with HAP SDF 5 (817 ± 360) compared with control groups Intact (367 ± 163) and Cavity (441 ± 257) (Figure 7C).

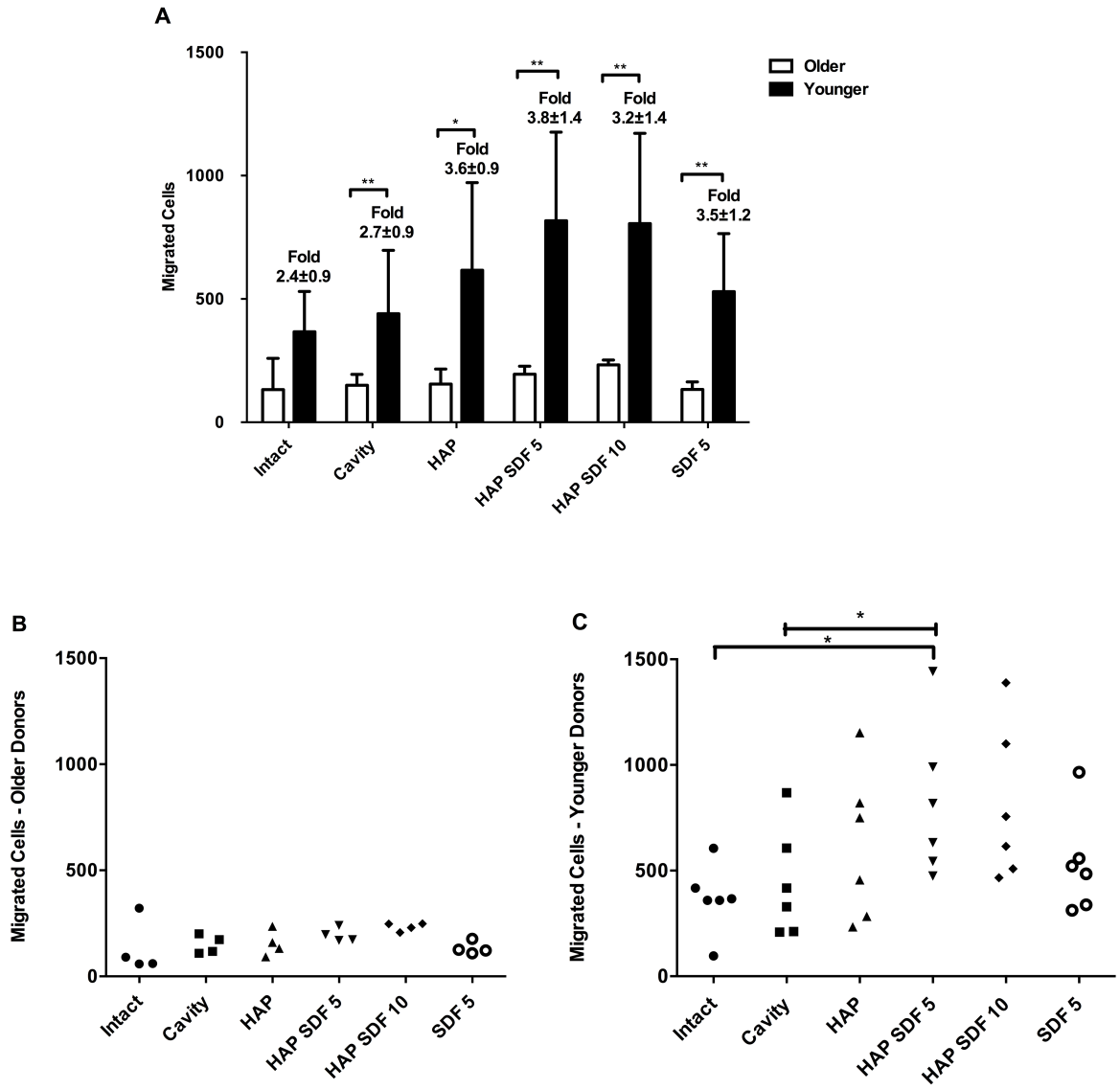

Figure 7 | hMSC migration in the *ex vivo* IVD cultures. The raw number of migrated cells for all hMSC donors compared for each study group: Intact, Cavity, HAP, HAP SDF 5, HAP SDF 10, and SDF 5. **A) Comparison of younger and older donors**: the impact of age in hMSC migration was evaluated by grouping the older (n = 2 human donors, n = 4 bovine tails) and younger (n = 3 human donors, n = 6 bovine tails) hMSC donors. hMSC migration was significantly higher for the younger donors in all the study groups, with the exception of the Intact group. Statistical significance was considered for p value (*) <0.05 (Mann–Whitney test). **B) hMSC migration in older donors**; **C) hMSC migration in younger donors**. Cell migration of older donors was not statistically different between different groups, contrarily to younger donors, where hMSC migration significantly increased from control groups (Intact and Cavity) to HAP SDF 5 (Friedman test, *, $p < 0.05$).

Effect of donor age on the migration profile of hMSCs in the ex vivo IVD organ culture model

We further addressed the migration profile of hMSCs along the disc depth. IVDs were cut sagittally allowing cell counting in sub-sections at distinct depths (as schematically depicted in Figure 2). The results for younger (Figure 8A) and older (Figure 8B) hMSC donors were analyzed separately and are represented as the mean cell number per sub-section in the groups: Intact, Cavity, HAP SDF 5 and HAP SDF 10. The other two groups (HAP and SDF 5) were also analyzed, but no statistically significant differences were observed (data not shown).

Concerning the migration profile for hMSCs from younger donors, the number of migrated cells generally decreased with the disc depth, while overall the number of cells were considerably increased in the HAP-SDF-1 groups (HAP SDF 5 and HAP SDF 10). Looking into the sub-sections, a significantly higher (*, $p < 0.05$) number of cells were observed in the first sub-section, S1, in HAP SDF 10 when compared to the Intact group (1.8-fold). In the following sub-section (S2) no differences were found between the conditions tested, although HAP-SDF-1 groups present also higher cell numbers than the controls. In the middle sub-sections (S3, S4) the effect of SDF-1 in the enhancement of cell recruitment was more evident, with higher numbers of hMSCs being detected in these sub-sections in HAP SDF 5 and HAP SDF 10 groups (*, $p < 0.05$). This trend was also noted in the following sub-section (S5) for the HAP SDF 10 group. Significant differences were also observed between the Intact or Cavity and the HAP SDF 10 groups in the section S6. In the last sub-sections (S7), cell migration was similar in all the groups.

Concerning the hMSC migration profile from older donors, a decrease in the total number of migrated cells with the disc depth was also observed. Contrary to the younger donors, no significant differences between the groups were observed in the first sub-section (S1). However, in the following section (S2), we could detect a significantly higher number of cells (*, $p < 0.05$) in Cavity and HAP SDF 10 *vs* Intact groups. This observation suggests that cell recruitment stimulated by SDF-1 was similar to the one triggered by the injury itself. Then, in all the following sub-sections (S3, S5, S6) no significant differences were observed between different groups with the exception of the intermediate sub-section S4 and the last section (S7), in which the effects of SDF-1 were more pronounced.

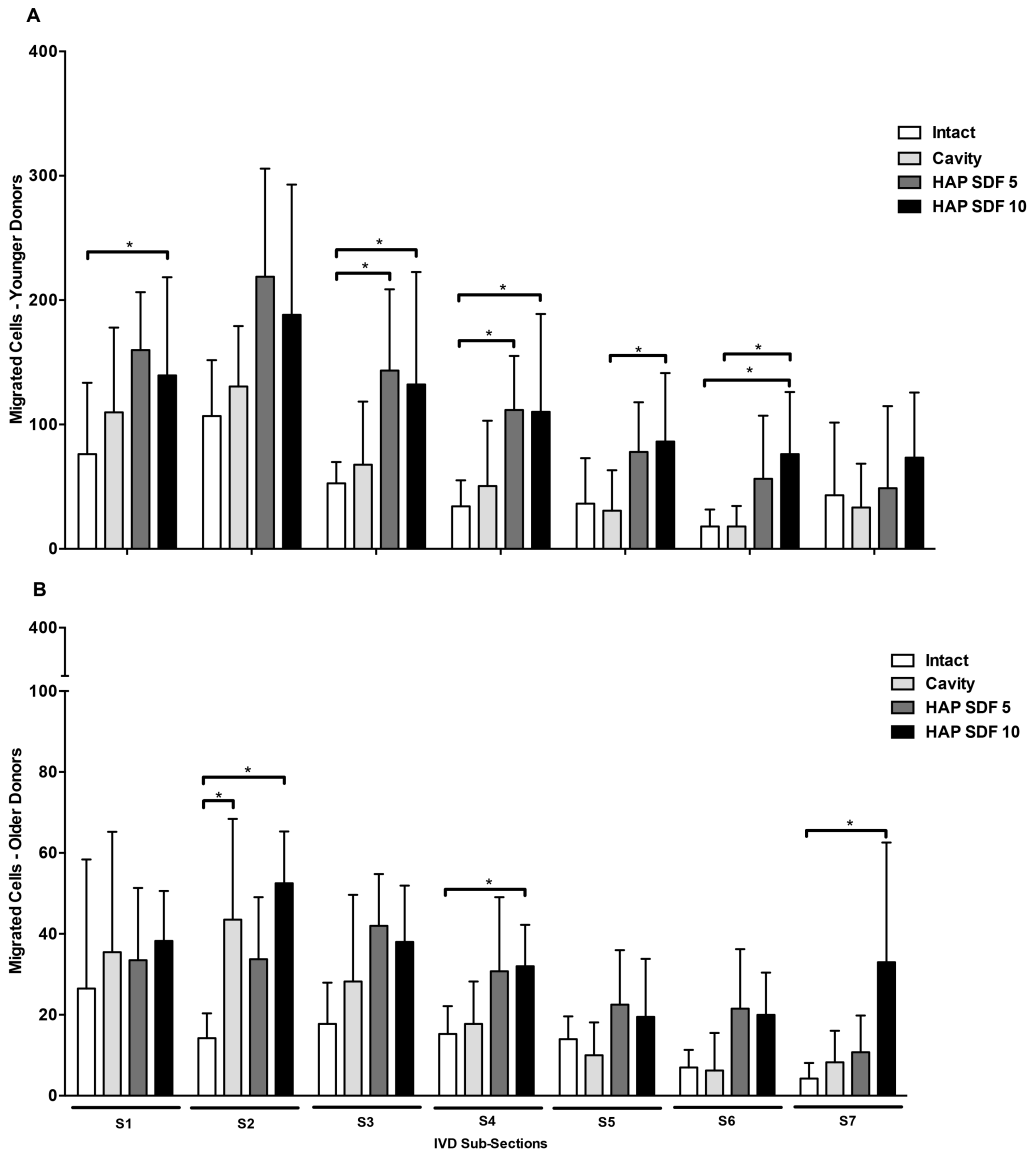

Figure 8 | hMSC migration profile in the *ex vivo* IVD cultures. The depth of migrated cells was analyzed in the groups of interest: Intact, Cavity, HAP SDF 5 and HAP SDF 10 for the younger (A) and older (B) hMSC donors. The Mean ± StDev of the number of cells found in each tested group (older: *n* = 2 human donors, *n* = 4 bovine tails; younger: *n* = 3 human donors, *n* = 6 bovine tails) is represented in the *y*-axis for each sub-section of disc height (represented in *x*-axis). The S1 section in the (*x*-axis) corresponds to the area surrounding the CEP where cells were seeded. Subsequent sub-sections correspond to those indicated in the scheme of Figure 2. Only the groups Intact, Cavity, HAP SDF 5 and HAP SDF 10 are represented (Statistical significance was considered for *p* value (*) <0.05 (Mann–Whitney test).

DISCUSSION

The present study aimed to investigate a chemoattractant-based delivery system for the recruitment of hMSCs towards a degenerating IVD. The capacity of hMSCs to migrate to injured tissues has previously been described[39,40,41], and several growth factors/chemokines have been identified as chemoattractants for hMSCs[42]. SDF-1 has been reported as an efficient chemoattractant for hMSCs, for example in bone fracture sites[43,44], or incorporated into biomaterial-based delivery systems, such as in Chitosan/Poly-γ-glutamic acid multilayer films[27]. Here, the incorporation of SDF-1 into a thermoreversible hydrogel (HAP) suitable for application in the IVD is described. The chemokine incorporation was performed by reconstitution of lyophilized HAP with an SDF-1 solution. We were able to successfully entrap and release SDF-1 from the hydrogel without chemical or physical modifications. *In vitro*, SDF-1 was rapidly released in the first 6 h and slowed down thereafter, reaching a maximum of 54% released after 7 days. The non-quantitative release of SDF-1 is expected and has widely been observed with other growth factors released from hydrogel matrices. This *in vitro* release system is not able to mimic the complexity of the *in vivo* environment, where the material is subjected to ECM components, protein interactions, immune response, and catabolic enzymes[31,33]. Therefore, the time scale and bioavailability of SDF-1 can differ significantly in presence of cells or *in vivo*. On the other hand, data obtained confirm the capability of HAP of slowly releasing SDF-1, preserving its structure and epitope as revealed by ELISA.

The potential of SDF-1-hydrogels to recruit hMSCs was evaluated in an *ex vivo* organ culture model using a nucleotomized IVD. Explant models represent an important alternative to *in vivo* studies, since whole-organ cultures allow the study of specific mechanisms in a more physiological environment than standard *in vitro* cultures. These explant cultures maintain IVD cells in conditions that are close to an *in vivo* scenario (hypoxia, limited nutrition, etc.)[45]. Concerning the use of bovine caudal discs, discs from bovine origin are known to have anatomic proportions and nutrient transport conditions similar to human lumbar discs[46]. Different *ex vivo* models for cell or hydrogel delivery in bovine discs have been described, including papain digestion of the NP[47] and NP cavity trough the CEP[33]. In our study, we adopted the cavity approach that uses nucleotomized healthy discs. This model presents better reproducibility of the defect created and facilitates the injection of hydrogels or solutions into the IVD. It also mimics the loss of NP tissue that occurs during degeneration, whereas the cavity approach allows the AF to remain intact during the procedure[32].

In this study, the cavity itself stimulated hMSC recruitment, although to a low extent. The proportion of recruited cells could then be effectively augmented by HAP-SDF-1 delivery systems, while soluble SDF-1 showed no effects. This result suggests that the soluble protein might be more prone to degradation in the tissue. SDF-1 release from HAP hydrogels

in *ex vivo* IVD cultures was confirmed by ELISA, although in lower concentrations than expected, which suggests that the protein might remain in the gel or become entrapped in the tissue. In addition, the maximum release of SDF-1 was observed at 24 h, which might indicate that the released chemokine was consumed or degraded. Future work will be performed to highlight the SDF-1 fate within IVD tissue. Nevertheless, the released chemokine was able to efficiently recruit hMSCs.

hMSC migration was similar in both NP and AF in all the groups studied, indicating that ECM pervasion by these cells is possible in both tissues. Typically, cell migration occurs via ECM degradation by matrix metalloproteinases (MMPs) and other proteolytic enzymes[48,39]. It is known that in induced degenerating IVDs, MMP13 and MMP7 are up-regulated[22]. Furthermore, *in vitro* studies of hMSC recruited by SDF-1 showed MMP-2 and membrane-type I matrix metalloproteinase (MT1-MMP) overexpression[50]. The latter was also identified in chondrocytes migrating from the cartilage endplate to the IVD[51]. Therefore, it will be of interest to explore the mechanisms regulating hMSC invasion through the IVD in future studies.

Herein, we addressed the effect of hMSC donor age on cell migration. It is known that the potential of hMSCs with identical phenotype is donor-dependent and passage-related[52,53]. Both aspects are critical to achieve a good clinical outcome. Our results indicate that hMSCs isolated from young donors present a significantly higher migration capacity, when compared to cells from older donors. This could partially be explained by the loss of expression of CXCR4, the most recognized receptor for CXCL12/SDF-1 ligand, with aging[54]. Recent literature also describes increased expression of adhesion receptors (melanoma cell adhesion molecule (MCAM/CD146) and vascular cell adhesion molecule-1 (VCAM-1)) in younger versus older hMSC donors, which could then influence cell migration capacity[38]. On the other hand, we cannot exclude the possibility that hMSCs migrate at reduced velocity with aging.

Finally, we analyzed hMSC migration profile within IVD depth. The migration profile shows random migration in the sub-section(s) closer to the CEP, suggesting migration by gravity and/or triggered by the injury itself. In the middle and deeper sub-sections, cell migration is clearly enhanced by HAP-SDF-1 hydrogels. Again, SDF-1 effect was more pronounced in hMSCs from younger donors, with higher numbers of cells being detected in the deeper sections.

The concept of cell migration in cartilaginous tissues has long been considered an unattainable goal, in part due to the avascular nature and low replication/migration capacity of the cells within the tissue[55,56]. This concept was challenged when regeneration of a synovial joint was achieved by homing of endogenous cells[57]. Recently, Zhang et al. explored MSC-conductive scaffolds containing SDF-1, achieving very promising results by improving *in situ* self-repair of partial-thickness cartilage defects in rabbits[58]. In the IVD, Illien-Jünger et al. previously demonstrated that a degenerating IVD by itself stimulates hMSC, but not fibroblast,

recruitment[22]. Moreover, Henriksson et al. suggested the existence of a stem cell niche in the outer AF region in an area adjacent to the epiphyseal plate[59], and Sakai et al. described NP progenitor cells in the Tie2 and GD2 positive cell populations in mouse and human IVDs, which show high proliferation capacity and are clonally able to differentiate into multiple mesenchymal and NP lineages[60]. Together, these recent evidences support the hypothesis of an intrinsic regenerative potential in the IVD, which opens a space for new therapies focused on cell targeting, activation and recruitment.

This study is limited to an *ex vivo* model and the use of healthy nucleotomized discs to mimic degeneration. Nevertheless, our model presents high reproducibility and overcomes the main drawback of the availability of human or animal naturally degenerated discs. Future work will focus on understanding hMSC migration mechanisms, while elucidating the contribution of hMSC recruitment to IVD regeneration.

CONCLUSIONS

This study demonstrates that the thermoreversible HAP hydrogel is a suitable carrier for SDF-1 delivery in the IVD, being able to recruit hMSCs towards an injury in the tissue. Importantly, HAP can also provide an inductive and conductive environment for the recruited cells, as this hydrogel has been shown to promote the NP-like phenotype. In addition, hMSC donor age could be a critical parameter for the success based on cell migration. The results presented here constitute a proof of concept for an innovative therapy for IVD regeneration aiming to enhance the tissue's endogenous repair capacity.

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CHAPTER VI

CHAPTER VI - ARTICLE 3

THE EFFECT OF HYALURONAN-BASED DELIVERY OF STROMAL CELL-DERIVED FACTOR-1 IN INTERVERTEBRAL DISC MATRIX REGENERATION

In preparation

The effect of hyaluronan-based delivery of stromal cell-derived factor-1 in intervertebral disc matrix regeneration

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ABSTRACT

The intervertebral disc (IVD) degenerative process is characterized by a deregulation of cell catabolic/anabolic activity and cell death, resulting in loss of native extracellular matrix (ECM) components and water. Repopulating the IVD with cells that could revert this process and recover the tissue homeostasis would be a major achievement. In the current study, the potential of a hyaluronan (HA)-chemoattractant delivery system to recruit Mesenchymal Stem Cell (MSC) from the cartilaginous endplate (CEP) and accelerate the regenerative process was explored. A HA-chemoattractant delivery system containing stromal cell derived factor-1 (SDF-1) (5 ng/μL) (HAPSDF5) was injected in the empty cavity of a nucleotomized bovine disc. MSCs were seeded in the CEP and let to migrate during different time points: 7, 14 and 21 days. At each time point, metabolic activity (rezasurin assay), DNA content and ECM production, namely collagen type II and aggrecan, of IVDs in culture were evaluated. In culture supernatants, SDF-1 and pro-regenerative growth factors (TGF-β1, bFGF) were quantified during time by ELISA. Nucleotomized discs (cavity), with or without MSCs, or with the delivery system HAPSDF5 by itself, were used as controls.

SDF-1 release peak was detected in the IVD culture medium 2 days after treatment, and only residual values were detected in further time points. Fluorescently labelled (CM-DiI)-MSCs migrated from CEP towards the IVD, remaining viable, even after 21 days in culture. HAPSDF5 increased the number of CM-DiI-MSCs migrating in the tissue in early time points, especially at day 14. The higher MSCs recruitment induced by HAPSDF5 appears to be related with an acceleration of ECM production in nucleotomized IVDs, namely collagen type II (col type II) at 14 days of culture, and increased production of pro-regenerative growth factors such as TGF-β1. These findings suggest that this chemoattractor-delivery system ability to enhance cell migration may accelerate IVD regeneration.

INTRODUCTION

The intervertebral disc (IVD) degeneration is a major contributor to spine-related pain[1]. Back pain is estimated to affect 84% of the population at some point of their lives[2]. The current clinical solutions, although alleviate symptoms, are often insufficient to effectively resolve the pain and the underlying problem, that in more than 40% of the back pain cases is the degenerated IVD[1].

IVD degeneration causes are multifactorial, although both the aging process and the genetic background have been described has the main contributors[3,4]. It's a progressive process, in which the tissue undergoes several histomorphological and biomechanical changes as a result of several disrupting events[5,6]. Among others, one of the hallmarks of the degenerative process is the increased cell death and senescence[7,8], that finally alters the whole IVD metabolism by decreasing the synthesis rate of the main matrix components, collagen type II (col type II) and aggrecan (agg)[9,10].

Boosting IVD cells metabolism or repopulating the IVD tissue with new cells have been proposed as alternatives strategies to the current surgical approaches which do not take into consideration the IVD biological function. This has been attempted by different approaches based on cell transplantation[11], injection of growth factors[12] or even genetic modifications[13], that demonstrated beneficial effects in IVD regeneration.

Cell-based therapies namely using mesenchymal stem/stromal cells (MSC), revealed to be particularly appealing, greatly due to the known multi-differentiation and immune-modulatory potential[14]. MSCs have the capacity, not only to migrate to injury sites in response to the released chemokines, but also of assuming different roles in the tissue, as cell empowerment, by the secretion of growth and immunomodulatory factors, or as cell replacement, by the differentiation into the native cells[15]. MSCs have also been shown to differentiate into disc-like cells and to contribute for IVD regeneration, as demonstrated in several previous studies[16-19].

Recently, stem cells recruitment to sites of injury has been the target of alternative therapeutic strategies aiming to stimulate tissue endogenous repair. This approach has been applied in different contexts, such as heart[20], bone[21,22] and cartilage regeneration[23], using chemoattractants molecules, namely stromal cell derived-1 (SDF-1). In the IVD scenario, cell recruitment has been poorly explored, namely due to the IVD nature and reduced repair capacity. However, in the past years, different findings have shed some light on the possibility to stimulate IVD endogenous repair. Henriksson et al. demonstrated the presence of progenitor cells in IVD surroundings and proposed a migration route for these cells towards the disc[24,25], while others have demonstrated the presence of progenitor cells within the disc itself[26,27]. In 2012, Illien-Junger and colleagues reported MSCs migration in the IVDs cultured under

degenerative conditions for the first time[28]. Later on, CCL5/RANTES was described as a key chemoattractor in this process with increased levels in both *ex vivo* IVD cultures[29] and in the plasma of patients with lumbar disc degeneration[30]. These findings unlocked a new line of research in the treatment of IVD degeneration, exploiting chemoattractants as a way to stimulate endogenous cells migration towards the IVD. Following this, our group has explored the ability of human MSCs to migrate from the CEP towards the disc in an *ex vivo* model of bovine IVD nucleotomy. This strategy was shown to impair IVD degeneration through ECM remodelling[31]. Furthermore, our group also shown that injection of a chemoattractant hyaluronan (HA)-based delivery system containing SDF-1 significantly enhanced hMSCs migration to the IVD[32]. Herein we aim to understand whether SDF-1-HA-based delivery system can improve IVD regeneration, either by accelerating and/or stimulating the ECM synthesis.

MATERIALS & METHODS

HA-based delivery system for SDF-1

HA Sodium salt from streptococcus equi was purchased from Contipro Biotech s.r.o. (Czech Republic) with a weight-average molecular weight Mw ¼ 1.5 MDa and polydispersion index ¼ 1.53. Amino-terminated poly (N-isopropylacrylamide) (pNIPAM-NH2) of 44 ± 2.7 kDa was purchased from Polymer Source, Inc (Canada). HAP was prepared by direct amidation reaction of thermoreversible segments of pNIPAM-NH_2 on HA backbone, as previously described by D'Este et al.[33]. The polymer solution was filter-sterilized, lyophilized and then reconstituted in PBS (pH 7.4) at a concentration of 10% wt/vol, containing 5 ng/μL of chemokine SDF-1 (Peprotech, UK) as previously described[32]. SDF-1 release from the hydrogel was assessed in the culture medium at different time points (48h, day 7, day 14 and day 21) by Enzyme-Linked Immunosorbent Assay (ELISA) (Human CXCL12/SDF-1 ELISA Development kit, R&D Systems, USA), following the manufactures' instructions.

Human Mesenchymal Stem Cells Culture

hMSCs were obtained from human bone marrow tissues donation from patients undergoing total hip arthroplasty or knee joint surgery. Patients gave informed written consent for tissue use for research purposes and procedures were carried out in accordance with the relevant guidelines approved by the Centro Hospitalar São João Ethics Committee. All samples were analysed with patient data coded. hMSCs were obtained from three different donors with ages of 45, 56 and 21 years and isolated by density gradient centrifugation and adherence to tissue culture plastic as previously described[34]. hMSCs were expanded in low-glucose Dulbecco's modified Eagle medium (DMEM, Gibco) containing 10% FBS and 1% Penicillin/Streptomycin (P/S) until reaching the total number of cells needed (1×10^6 cells/disc), in general in passages P5 to P6.

Intervertebral disc isolation

Bovine IVDs were isolated from young adult animals' tails (5–10 months old) within 3h post-slaughter in a local slaughterhouse (Carnes Landeiro, Barcelos, Portugal). All experiments were performed in accordance with relevant guidelines and regulations, with the ethical approval of the Portuguese National Authority for Animal Health. IVDs with CEPs were harvested in sterile conditions following a protocol previously described[32]. Briefly, the caudal discs with CEPs were removed using a band saw (Dremel ® Moto-Saw (MS20-1/5)) to obtain parallel cuts. The CEPs were afterward jet-lavaged with sterile phosphate-buffered saline solution (PBS, pH 7.4), using a Pulsavac wound debridement irrigation system (Zimmer, Inc., Switzerland). Discs were washed sequentially in 1%/10%/1% of P/S in PBS (pH 7.4) for 1/10/1

min, respectively. Afterwards, discs were incubated overnight in 6-well plates with high-glucose Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 2% fetal bovine serum (FBS, Gibco), 1% P/S (Gibco), 1% insulin transferrin selenium supplement (ITS) (BD, Becton Dickinson) and 0.1% Primocin (Invivogen) at 37°C in a 5% CO_2 atmosphere.

Ex vivo IVD organ culture

A previously established IVD organotipic culture was used in this study[17,31,32], where mechanical induced nucleotomy was performed to establish a degenerative condition. Briefly, a circular cavity was performed through the CEP and part of the NP (0.05-0.1 cm^3 of tissue) was removed using a blade. The cavity was afterwards filled with the hydrogel or remained empty. Right after CEP reposition, the lesion area was sealed with bone cement (PMMA, Vertecem V Cement Kit, Synthes, Switzerland). Finally, all the discs were turned to place the cavity at the bottom of the well and incubated in medium (DMEM 4.5 g/L glucose, 2% FBS, 1% Pen/Strep, 1% ITS and 0.1% Primocin) in 6-well plates for 2 h before hMSCs seeding.

In this study, four groups were defined (Figure 1):

a) Cavity (disc with lesion and empty caviy);

b) C+hMSCs (disc with an empty cavity in which hMSCs were seeded in the CEP);

c) C+HAPSDF5+hMSCs (disc with cavity injected with HA-based delivery system containing SDF-1 (HAPSDF5) plus hMSCs seeded in the CEP;

d) C+HAPSDF5 (disc with injected HAPSDF5, without hMSCs).

In the discs with hMSCs co-culture, cells were afterwards seeded at a density of $1x10^6$ cells/disc, on the top of the CEP (i.e. on the side opposite to the injury) and incubated for 30 min to allow cells to adhere to the CEP structure (Figure | 1). Afterwards medium (DMEM 4.5 g/L glucose, 2% FBS, 1% Pen/Strep,1% ITS, 0.1% Primocin) was added and discs were cultured at 37ºC in a 5% CO2 atmosphere incubator during 7, 14 and 21 days. After each time point, discs were extensively washed with PBS and cut sagittaly using a Dremel ® Moto-Saw (MS20-1/5). The CEPs were afterwards removed and ½ of the disc was store at -20ºC for further analysis while the other half was fixed in 10% buffered saline for histology.

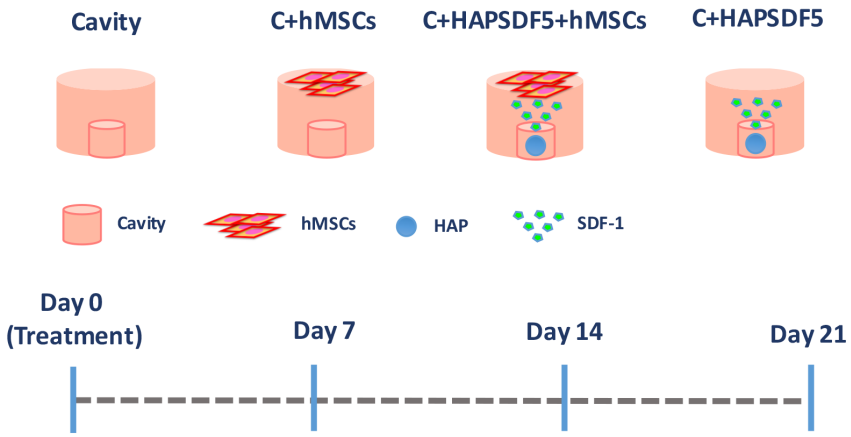


Figure 1 | Schematic representation of the experimental setup.

Metabolic activity and DNA quantification in the organ culture along time

The mitochondrial metabolic activity of IVD cells was evaluated at the different time points using the resazurin assay, as previously described[31]. Cell metabolic activity was expressed as relative fluorescent units and normalized to the wet weight of the tissue (mg). A blank control comprising only medium was also included. Resazurin conversion was measured using a Synergy™ Mx multi-mode microplate reader (BioTek® Instruments, Inc., Vermont, CA) with 530 nm excitation filters and 590 nm emission filters.

DNA content in the IVD tissue was quantified using a CyQuant® kit (Invitrogen). Briefly, previously frozen IVD samples were minced into very small pieces and then digested in a proteinase K solution (0.5 mg/mL in phosphate buffer containing 10.68 g/L $NaH_2PO4.2H_2O$, 8.45 g/L $Na_2HPO_4.7H_2O$ and 3.36 g/L Disodium-EDTA in ultrapure water, pH 6.5) overnight at 56ºC. After vortexing, the cellularity of each sample was measured based on the DNA content using above mentioned kit with lambda DNA as a standard. DNA content was expressed as relative fluorescent units and normalized to the wet weight of the digested tissue.

Extracellular Matrix components assessment along time

The most abundant ECM components of the NP – col type II and agg, were evaluated at day 7, 14 and 21, to assess the effect of HAPSDF5 in matrix remodelling. Col type II quantification and expression in the IVD tissue was assessed both by western blot (WB) and afterwards by immunofluorescence (IF) to evaluate the distribution in the tissue.

For WB, part of previously frozen IVD was minced and incubated with an optimized buffer for protein extraction containing 4 M guanidine hydrochloride (Sigma), 3 M sodium acetate (Merck) and 10 mM EDTA, and enriched with a protease and phosphatase inhibitor cocktail (Roche Diagnostics GmbH, Manheim, Germany and Sigma, respectively). Protein quantification was performed using the 2-D Quant Kit (GE Healthcare) according to the

manufacturer's instructions. Protein samples (20 µg) were afterwards separated by sodium dodecyl sulfate (SDS)/9% polyacrylamide gel electrophoresis, and electroblotted onto a Hybond enhanced chemiluminescence (ECL) membrane (Amersham BioscienceseGE Healthcare). The monoclonal antibody against Col type II (1:1000 dilution) (II-II6B3) was used with a sheep anti-mouse (1:3000 dilution; Amersham Biosciences) horseradish peroxidase-conjugated secondary antibody, followed by ECL detection (Amersham Biosciences). Bands were quantified using Quantity One® 4.6.6 Software (Bio-Rad, Amadora, Portugal). Values were normalized to the density of each corresponding complete lane (total protein loaded)[35]. Results were afterwards normalized to the cavity group and presented as a fold increase (n=5-9). For Col type II distribution analysis by IF, antigen retrieval was performed in paraffin sections through incubation with a 20 µg/mL proteinase K (Sigma-Aldrich) solution for 15 min at 37 °C. After a blocking step, sections were then incubated for 2 h at 37 °C with the primary antibody against Col type II (1:50) (monoclonal antibody against Col type II (II-II6B3) developed by Dr. Thomas F. Linsenmayer, from the Developmental Studies Hybridoma Bank under the auspices of the NICHD and maintained at the University of Iowa, Department of Biology, Iowa City, IA 52242). Alexa Fluor 594-labeled goat anti-mouse (Invitrogen-Molecular Probes, 1:1000) was used as the secondary antibody for Col II detection during 1h, room temperature, in the dark. All sections were mounted in Fluorshield with DAPI (Sigma). Control sections for each immunolabeling excluded primary antibody staining. Representative images were taken using an inverted microscope, Axiovert 200 M, Zeiss. Col II intensity in the different regions of the IVD (NP and AF) was quantified using a custom-made software written in MATLAB (The MathWorks Inc., Natick MA, USA), IntensityStatisticsMask[31] (n = 5).

Agg expression was analysed by immunohistochemistry (IHC) in previously prepared tissue paraffin sections without CEPs, using the NovolinkTM Polymer Detection Kit (Leica Biosystems, Newcastle, UK) by following the manufacturer's instructions. Antigen retrieval for Agg was performed through the incubation with a 20 µg/mL proteinase K (Sigma-Aldrich) solution for 15 min at 37 °C. After neutralization of endogenous peroxidase using Peroxidase Block for 5 minutes, a blocking step was performed. Sections were afterwards incubated with the primary antibody Agg (H-300) sc-25674 (Santa Cruz Biotechnology, Inc, Texas, USA) (1:50) overnight. Bound antibodies were revealed after a 30 min incubation with NovolinkTM Polymer in the dark and 5 min incubation with peroxidase-substrate solution DAB. A negative control was performed in each slide without the primary antibody. Representative images of the slides were taken using an Olympus CX31 light microscope (20x objective for counting and 100x oil objective for detailed imaging of Agg IHC; 10x for Col I IHC). Agg matrix staining in the different regions of the IVD (NP and AF) was qualitatively assessed by two independent observers. Agg cellular staining was quantified using a custom-made software written in MATLAB (The MathWorks Inc., Natick MA, USA) ImmunoCellCount[31] (n = 5).

Growth factors Analysis in IVD culture media

The IVD organ culture medium was collected at different time points (day 7, 14 and 21), centrifuged (3000 rpm, 5 min) and kept at -20ºC for posterior analysis. Free active transforming growth factor beta (TGF-β1), basic fibroblast growth factor (bFGF) and Granulocyte-macrophage colony-stimulating factor (GM-CSF) were quantified in the medium by ELISA according to manufacturer's instructions (LEGEND MAX™Pre-Coated ELISA kits, Biolegend San Diego, CA).

Statistical Analysis

Results are presented as Median ± Interquartile Range (IQR) in box and whiskers plots. Statistical analysis was performed using Prism 5.0a for Mac OS.X. The parametric distribution of the data was evaluated by D'Agostino and Pearson normality test. For non-parametric data, when two groups were compared (e.g. cell migration) Wilcoxon matched-pairs test was used; when more than two groups were compared within non-related time points, Kruskal-Wallis test with Dunn's multiple comparison test was used. The two-away ANOVA with Tukey multiple comparison test was used in groups with related time points (e.g. SDF-1 ELISA). Statistical significance was considered at least for *$p< 0.05$, (**$p < 0.01$, ***$p< 0.005$, ****$p<0.001$).

RESULTS

SDF-1 delivery along time in the IVD culture

SDF-1 release from the HAP hydrogel injected in the cavity of a nucleotomized disc was assessed in the discs' culture media at different time points, day 7, day 14 and day 21, and additionally, 48h after treatment, as evaluated in our previous study[32] by ELISA. The results are presented as the Median ± IQR (Figure 2). The statistical analysis of the results, demonstrated an interaction (***p*=0.0045) of both variables time (*****p*<0.001) and the treatments performed (**p*=0.018), so determining the statistical analysis with the two-away ANOVA with Tukey multiple comparison test. At day 2, the average SDF-1 levels were residual in the cavity (0.26 ± 0.3 ng/mL) and C+hMSCs (0.26 ± 0.3 ng/mL) groups, where no exogenous SDF-1 was added, while, significant higher levels of SDF-1 were detected in both groups injected with HAPSDF5, C+HAPSDF5+hMSCs (0.43 ± 1.2 ng/mL; **p*<0.05) and C+HAPSDF5 (0.68 ± 1.2 ng/mL; **p<0.01), comparing to the cavity and C+hMSCs, respectively. At day 7, day 14 and day 21, the levels of SDF-1 were similar among the groups, despite at day 14, a slightly increase is observed in both SDF-1 containing hydrogel groups. Comparing the data through time, the levels of SDF-1 in the C+HAPSDF5+hMSCs group were shown to be significantly higher than all the groups (#) at day 7 (**p*<0.05), at day 14 (***p*<0.01) and day 21 (****p*<0.005). Similar trend could be observed in the C+HAPSDF5 group, where the levels found at day 2 were significantly higher comparing to all the groups at day 7 (***p*<0.01), day 14 (****p*<0.005) and day 21 (****p*<0.005). These results are in accordance with previous observations[32], and suggest that the SDF-1 effect may be limited to the first days of culture.

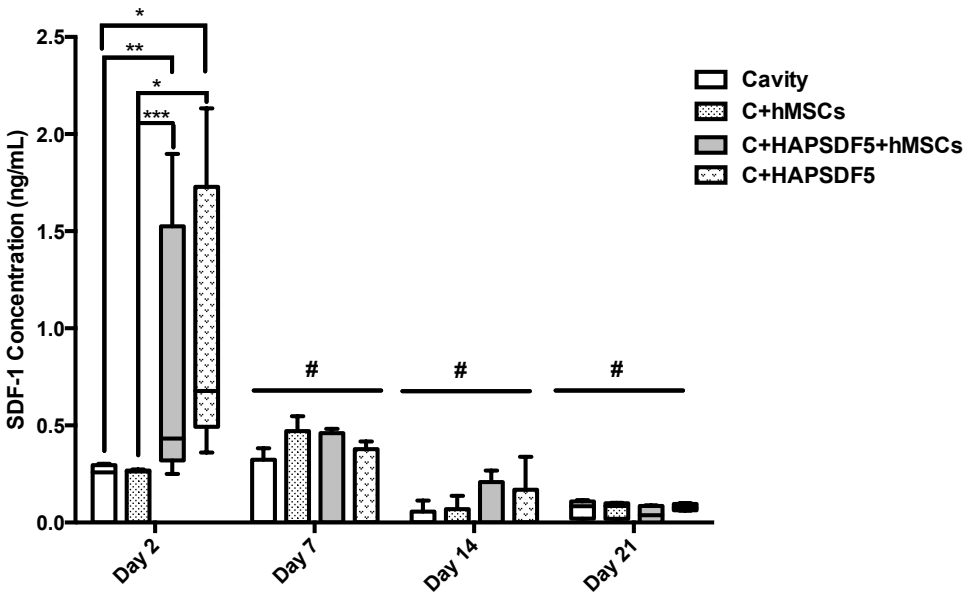


Figure 2 | SDF-1 Release from the HA-based delivery system in the IVD organ culture. SDF-1 release was assessed by ELISA in the organ culture media. Results are presented as box-and-whiskers plots (n=5). Statistical analysis was performed using two-away ANOVA, with Tukey's multiple comparison test (*p<0.05; **p<0.01, ***p<0.005).

Viability of the organotypic culture in different time points: Metabolic activity and DNA content.

Metabolic activity of the IVD cells was assessed at day 7, day 14 and day 21 in the different conditions by resazurin assay (Figure | 3). At day 7, a slight increase in metabolic activity can be observed in the C+hMSCs (2.4 ± 0.5 RFU/mg), C+HAPSDF5+hMSCs (1.9 ± 1.7 RFU/mg) and C+HAPSDF5 (2.6 ± 1.6 RFU/mg) groups, comparing to the cavity (1.6 ± 1.4 RFU/mg). At day 14, the cavity (2.2 ± 2.2 RFU/mg) and the C+hMSCs (2.3 ± 1.1 RFU/mg) groups presented similar metabolic levels, while a slight increase was observed in the C+HAPSDF5+hMSCs (2.7 ± 1.6 RFU/mg) and C+HAPSDF5 (3.0 ± 2.1 RFU/mg) groups. At day 21, the metabolic activity is very similar among the four groups: cavity (2.7 ± 1.6 RFU/mg); C+hMSCs (2.9 ± 2.6 RFU/mg); C+HAPSDF5+hMSCs (2.4 ± 2.1 RFU/mg) and C+HAPSDF5 (2.6 ± 2.9 RFU/mg). Despite the small variations of the metabolic activity, no significant differences were observed during the different time points or between each group/treatment, suggesting that in long term culture the metabolic activity could be maintained regardless of the injury/treatment.

The DNA content in each condition at the different time points has also been evaluated (Figure 4). At day 7, a higher DNA content can be observed in both groups containing

HAPSDF5 regardless of the presence of hMSCs (C+HAPSD5+hMSCs (0.07 ± 0.05 µg/mL) and C+HAPSD5 (0.06 ± 0.03 µg/mL)) compared to the cavity (0.04 ± 0.04 µg/mL) and the C+hMSCs (0.04 ± 0.04 µg/mL) groups. A similar trend was observed at day 14, with lower levels in the cavity (0.06 ± 0.01 µg/mL) and C+hMSCs (0.05 ± 0.01 µg/mL) groups, and higher ones in the HAPSDF5 containing groups C+HAPSD5+hMSCs (0.074 ± 0.02 µg/mL, *p<0.05) and C+HAPSD5 (0.068 ± 0.04 µg/mL). Finally, at day 21 the DNA content, was shown to be very similar among the groups: cavity (0.05 ± 0.03 µg/mL), C+hMSCs (0.06 ± 0.03 µg/mL), C+HAPSD5+hMSCs (0.05 ± 0.04 µg/mL) and C+HAPSD5 (0.06 ± 0.03 µg/mL). The higher DNA levels at early time point suggests that the presence of HAPSDF5 might trigger cell proliferation and/or promote MSCs migration to IVD.

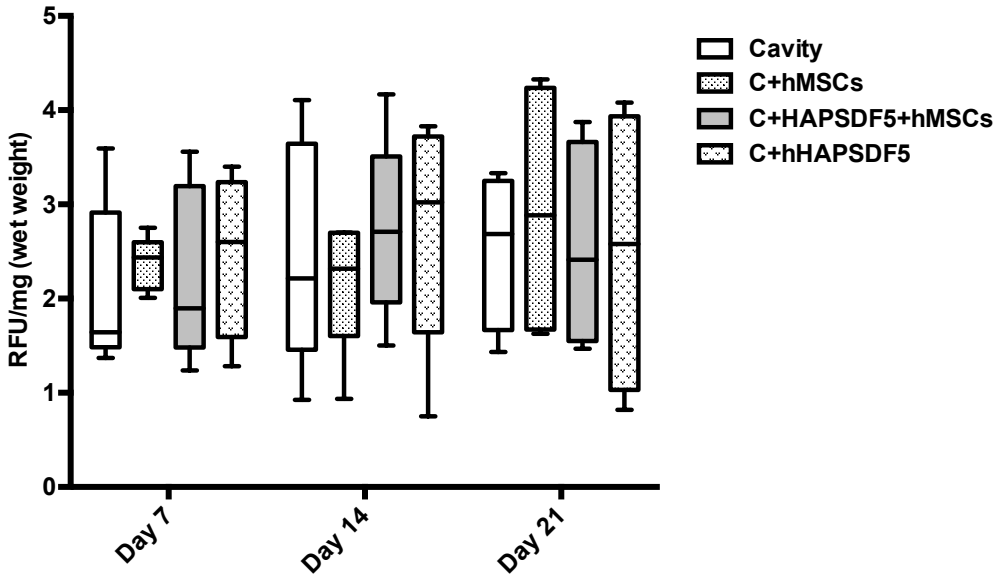


Figure 3 | Metabolic activity. Metabolic activity was measured in the discs upon incubation with 10% Resazurin solution. Results are presented as box-and-whiskers plots (n=5). Statistical analysis was performed using Kruskal-Wallis test with Dunn's multiple comparison. No significant differences were detected.

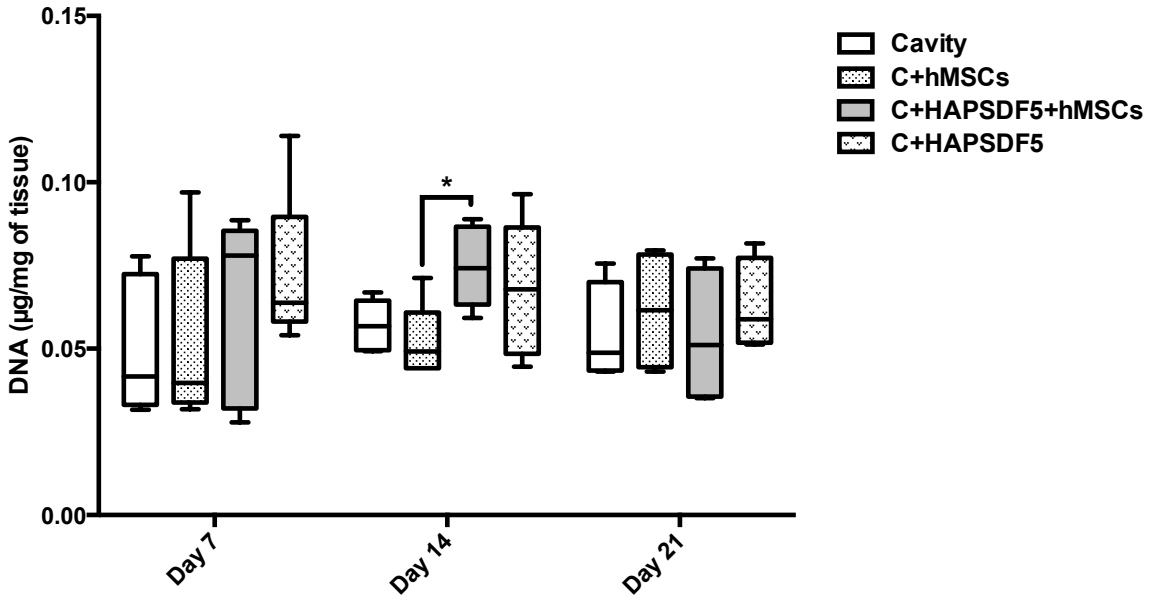


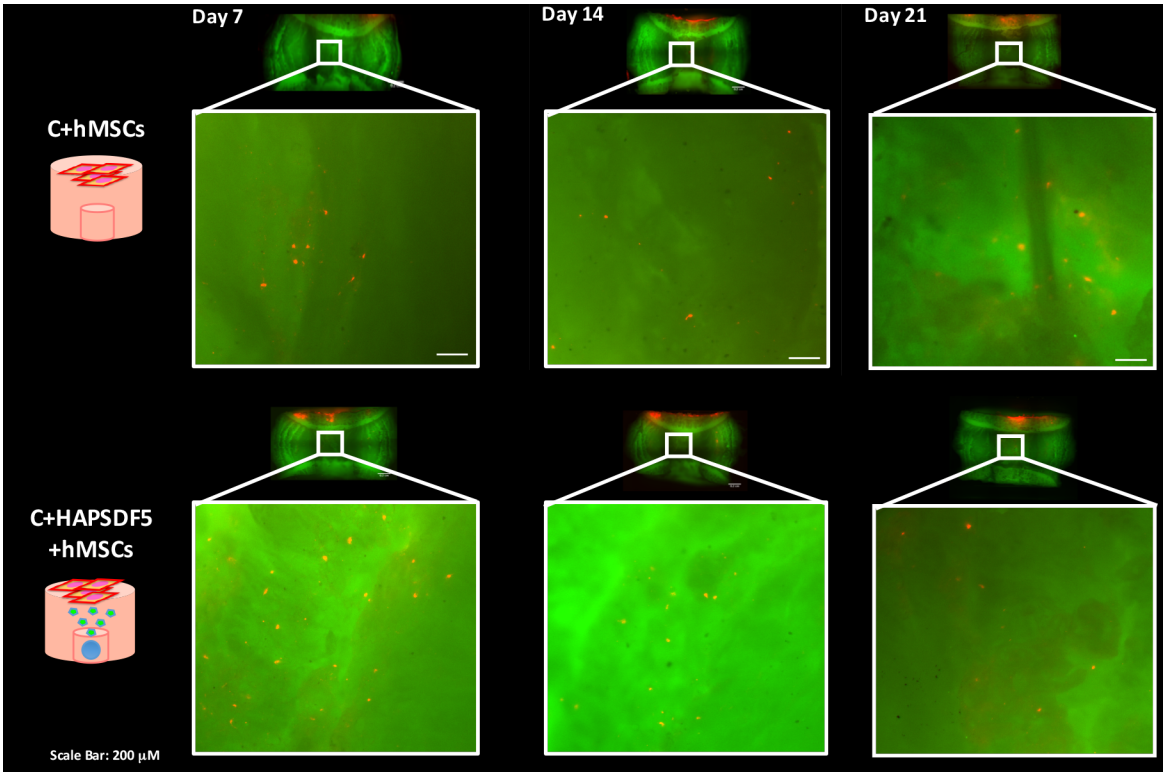
Figure 4 | DNA quantification in the tissue. Results are presented as box-and-whiskers plots (n = 5). Statistical analysis was performed using Kruskal-Wallis test with Dunn's multiple comparison. Significant differences in DNA content was observed at day 14 between C+hMSCs and C+HAPSDF5+hMSCs group (*p<0.05).

hMSCs migration profile at different time points

Previous studies have shown a significant higher recruitment of hMSCs from the CEP in the presence of the chemoattractant delivery system (HAPSDF5) 48h after administration[32]. To evaluate if the injection of HA-based chemoattractant delivery system containing SDF-1 (HAPSDF5) was triggering cell migration during the 21 days of culture, hMSCs were previously labelled with CM-Dil a fluorescent dye. Cell migration in the IVD tissue was assesses by microscopy in the IVDs central sagittal section from different animals, at the time points (Day 7, 14 and 21) using a high content screening system (InCell Analyzer 2000). CM-Dil-hMSCs migrating from the CEP could be identified and quantified in the sections (Figure 5A). Cell migration was compared in the groups containing hMSCs (C+hMSCs and C+HAPSDF5+hMSCs) (Figure 5B). A higher cell migration was observed in the group containing the HA-based chemoattractant delivery system in each time point, although only statistically significant at day 14 (*p<0.05), corroborating the previous observed trend on DNA content.

We have further analysed the migration profile of hMSCs along the disc depth, following the method described before[32]. The migration profile in both groups was similar in early time points, being the higher percentage of cells concentrated in the first two sections right after the

CEP, while at day 21, a higher migration into deeper sections of the IVD could be observed, suggesting that hMSCs continue to migrate along time. Although a slight higher percentage of migrating cells was present if the group containing HAPSDF5, this was not significantly different from the discs treated only with hMSCs (C+hMSCs) (Figure 5C).



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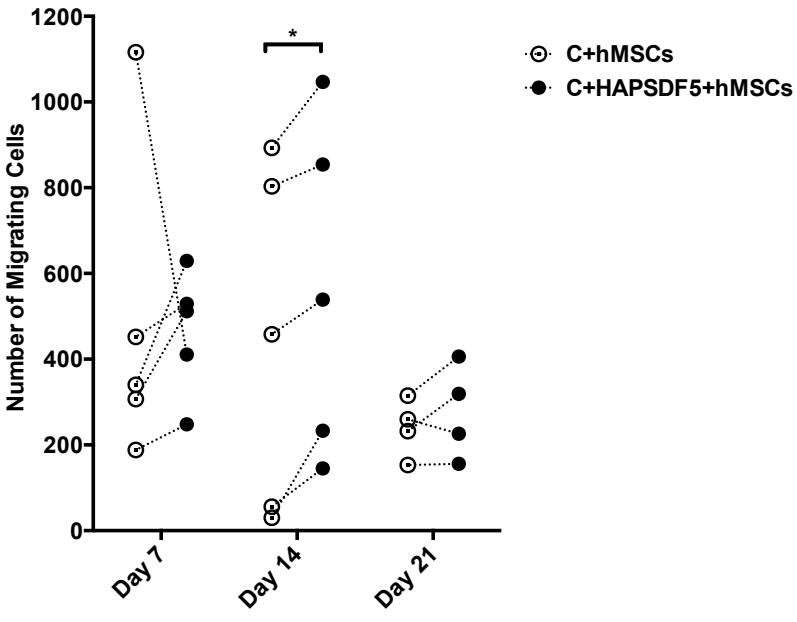

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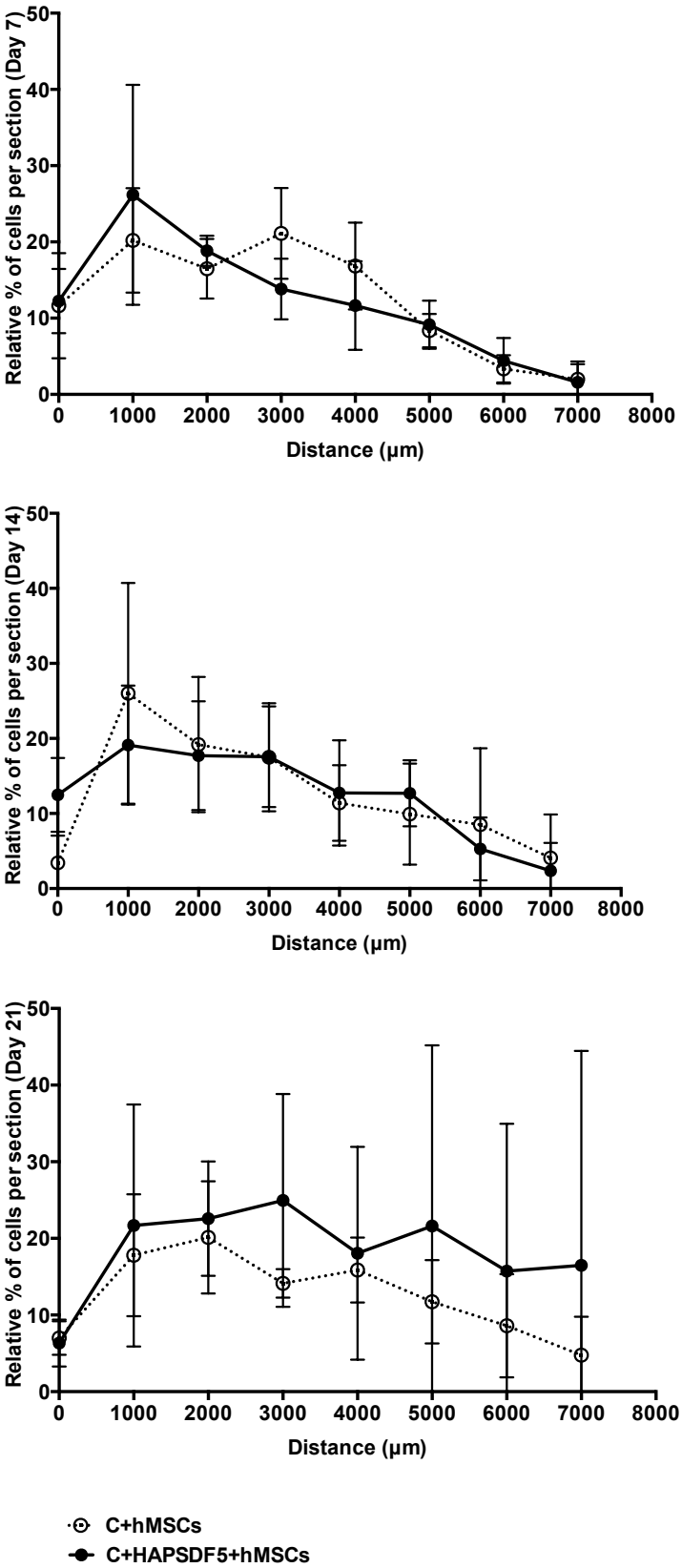

Relative % of cells per section (Day 7)
Distance (µm)
Relative % of cells per section (Day 14)
Distance (µm)
Relative % of cells per section (Day 21)
Distance (µm)
C+hMSCs
C+HAPSDF5+hMSCs

C

Figure 5 | hMSCs Migration in the IVD tissue. A | Representative images of IVD sagittal sections with CM-DiI labelled hMSCs at the different time points (Day 7, 14 and 21) in the C+hMSCs and C+HAPSDF5+hMSCs groups. **B |** Quantification of hMSCs migration in the IVD. Results are present as a plot of individual values (n=4-5). **C |** Migration profile of hMSCs along the IVD depth. Results are presented as XY plot (Y= % of cells/section; X=distance from the CEP towards the lesion) (n=4-5). An increased cell migration could be observed in the groups containing HAPSDF5, namely at day 14 (*p<0.05). Statistical analysis was performed using Wilcoxon matched-pairs test.

Effect of HAP/SDF-1 on ECM remodelling

Collagen type II expression

hMSCs migration from the CEP towards the centre of the IVD has been already shown to have a role in ECM remodelling[31]. Herein, the role of HAPSDF5 in accelerating this effect, by enhancing hMSCs recruitment was explored in early time points. Overall Col type II expression was quantified by WB in the IVD tissue collected at day 7, 14 and 21. The results were normalized to the respective control (cavity) and are presented as median ± IQR-fold. An increase in col type II expression can be observed in the C+HAPSDF5+hMSCs group (2.84 ± 5.6-fold) at day 7 when comparing to the C+hMSCs (1.0 ± 2.0-fold) and C+HAPSDF5 (0.86 ± 6.83-fold) at day 7, although not significant (Figure 6). At day 14, a significant increase in col type II expression was observed in the C+HAPSDF5+hMSCs group (2.9 ± 7.6-fold) comparing to group without the chemoattractant delivery system (C+hMSCs, 0.99 ± 0.9-fold; *p<0.05), and a slight increase was also observed comparatively to the HAPSDF5 alone (1.3. ± 1.4-fold; p=0.07) (Figure 6). While at day 21, although a higher expression of in col type II was observed in the C+hMSCs group (1.6 ± 1.4-fold), small variation was observed in the C+HAPSDF5+hMSCs (1.12 ± 1.15-fold) and C+HAPSDF5 (1.17 ± 0.98-fold) groups (Figure | 6). To further understand the distribution of this expression the IVD, IF of col type in IVD sections was performed. Col type II expression was common to the central NP and inner AF (Figure 7 | A), while no expression was found in the outer AF (data not shown).

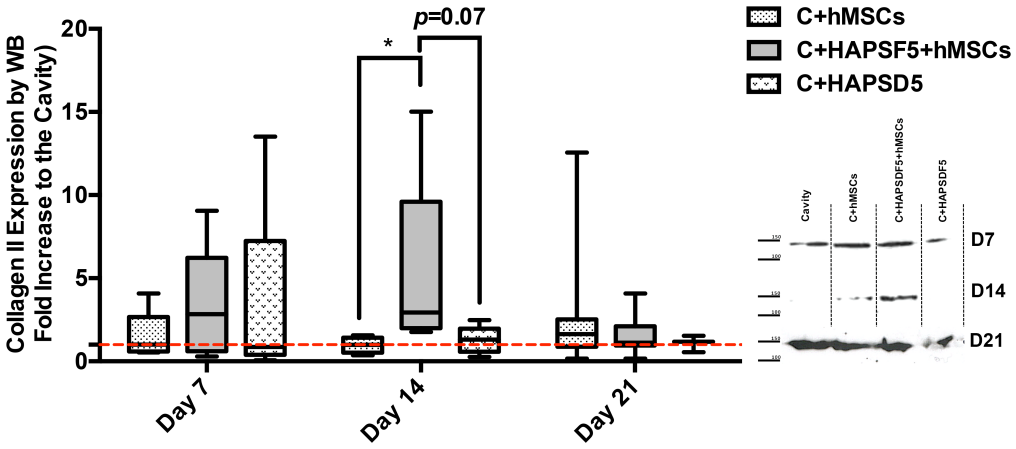
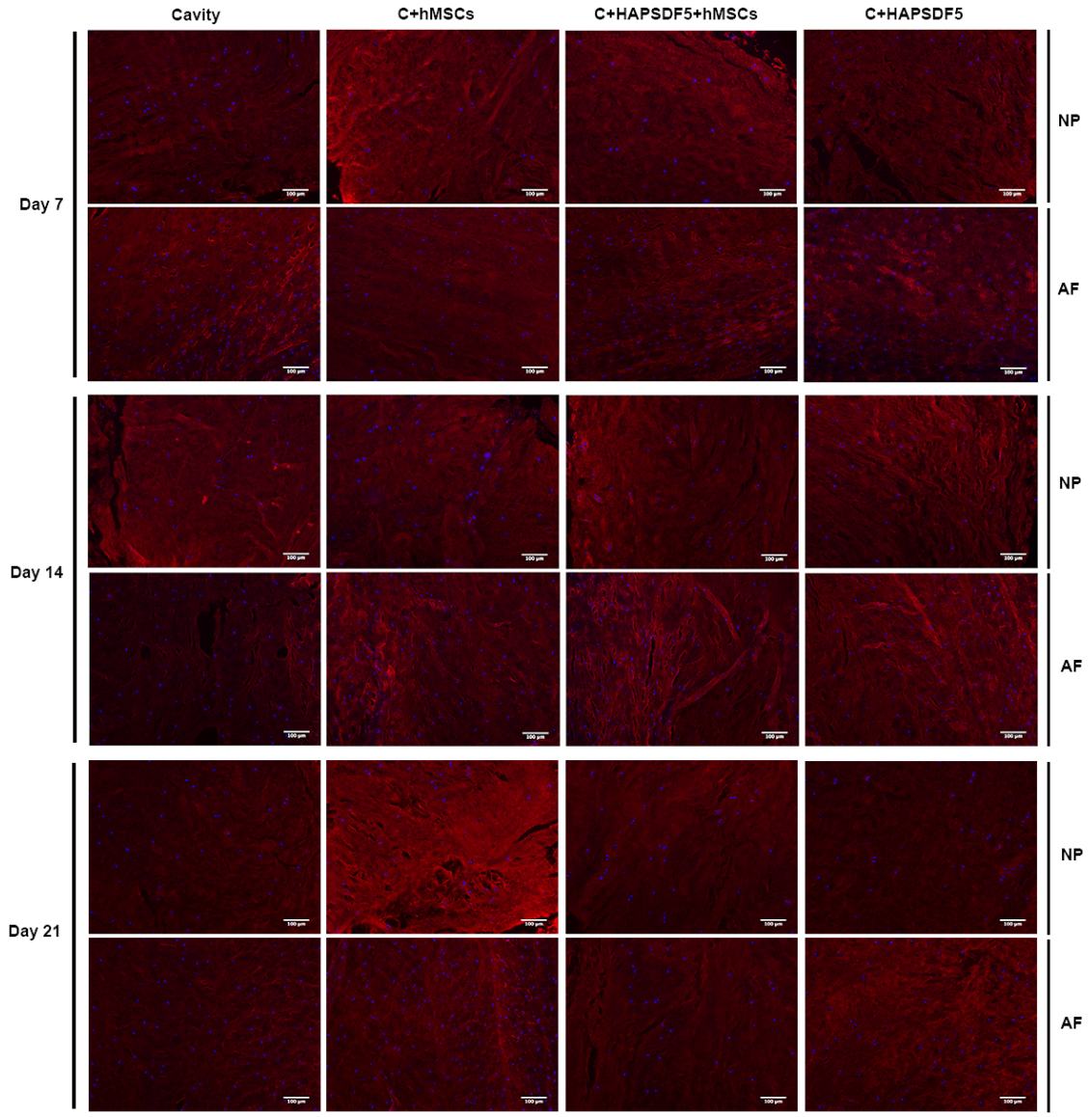


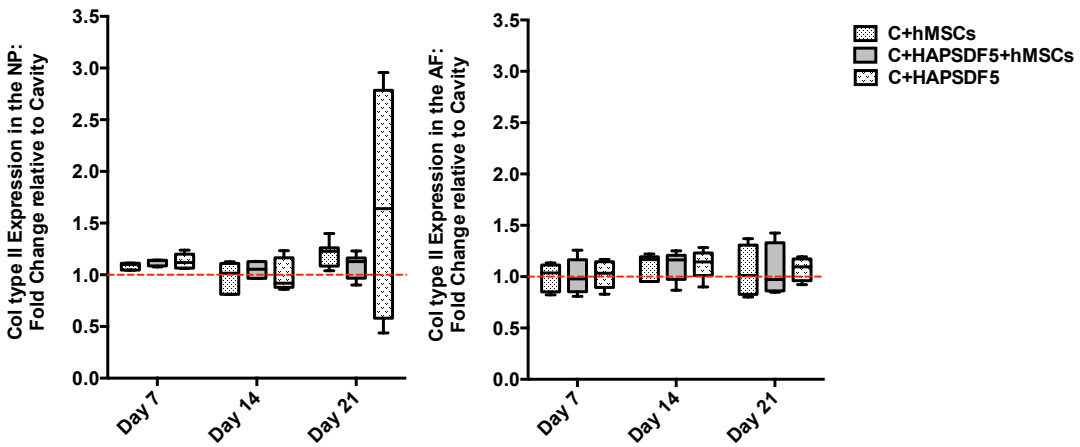
Figure 6 | Col type II analysis by WB. Col type II expression quantification in the IVD digested tissue by WB **and** representative images of the WB at the different time points. Results are normalized by the cavity control and presented as box-and-whiskers plots (n=5-9). Statistical analysis was performed using Kruskal-Wallis test with Dunn's multiple comparison. Significant differences were observed at day 14 between C+hMSCs and C+HAPSDF5+hMSCs group (*p<0.05).

This expression and distribution in the tissue was quantified both the NP/inner AF (Figure 7 | B). At day 7 there is an increase of col type II in the NP area, in the C+hMSCs (1.07 ± 0.06-fold), C+HAPSDF5+hMSCs (1.14 ± 0.05-fold) and C+HAPSDF5 (1.09 ± 0.09-fold) comparatively to the control (cavity), while in the inner AF the expression is very similar among the groups: 1.04 ± 0.26-fold; 0.99 ± 0.31-fold and 1,03 ± 0.25-fold, respectively. At day 14, a small increase in col type II expression was observed in the NP, in the C+HAPSDF5+hMSCs group (1.05 ± 0.16-fold), while the C+hMSCs (1,01 ± 0.30-fold) was relatively similar to the control and in the C+HAPSDF5 (0.92 ± 0.28-fold), a decrease was observed. In the inner AF, there was a slight increase in all groups comparatively to the control C+hMSCs (1.17 ± 0.24-fold), C+HAPSDF5+hMSCs (1.16 ± 0.24-fold) and C+HAPSDF5 (1.14 ± 0.22-fold).

At day 21, col type II expression was higher in the C+HSMCs group (1.17 ± 0.22-fold), in the NP, corroborating previous results[31], and also increased in the C+HAPSDF5+hMSCs (1.13 ± 0.20-fold) and the C+HAPSDF5 (1.64 ± 2.20-fold) groups, although with high variability in the latest. In the inner AF, at day 21, small variation was observed, both C+hMSCS (1.01 ± 0.48-fold) and C+HAPSDF5 (1.09 ± 0.21-fold) faintly increased from the control while decreasing in the C+HAPSDF5+hMSCs group (0.97 ± 0.47-fold). From the observed results regarding the col type II distribution, the higher expression in the C+HAPSDF5+hMSCs observed by WB at early time points (day 7 and day 14), seems to be related with a higher deposition of col type II in the NP area, although at day 14 the inner AF area might also contribute for the observed result.


Cavity
C+hMSCs
C+HAPSDF5+hMSCs
C+HAPSDF5
Day 7
Day 14
Day 21
NP
AF

A


Col type II Expression in the NP: Fold Change relative to Cavity
Col type II Expression in the AF: Fold Change relative to Cavity
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Day 7
Day 14
Day 21
C+hMSCs
C+HAPSDF5+hMSCs
C+HAPSDF5

B

Figure 7 | Col type II analysis in situ by IF. Col type II expression and distribution in the tissue was accessed by IF in sagittal sections of the IVD. **A |** Representative sections of col type II expression both in the AF and NP of the different time points. **B |** Fluorescence quantification in the NP and AF tissue. Results are normalized by the cavity control and presented as box-and-whiskers plots (n=5-9). Statistical analysis was performed using Kruskal-Wallis test with Dunn's multiple comparison.

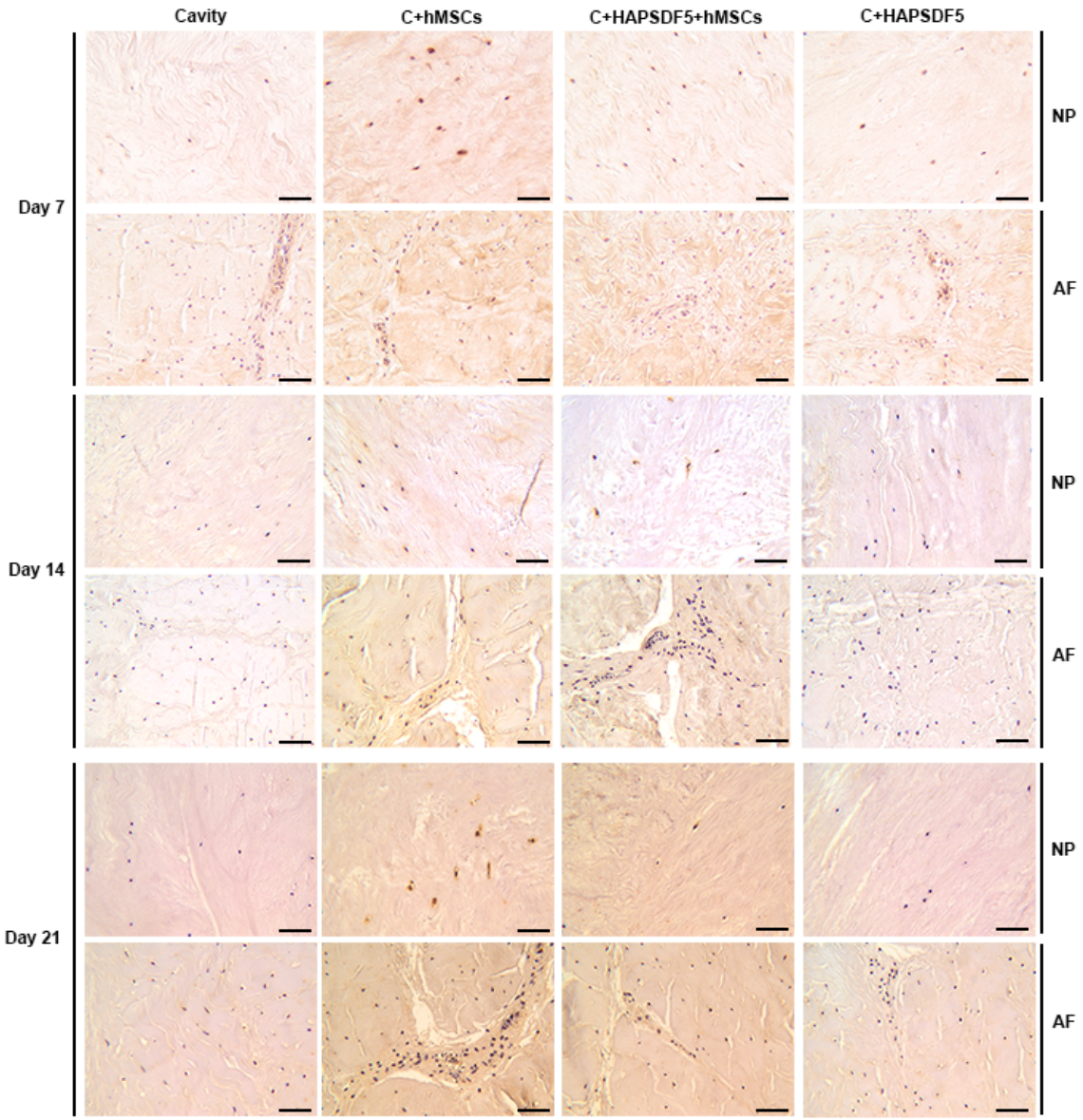
Aggrecan expression

Agg, the most abundant proteoglycan of the IVD has been also identified in the IVD tissue (Figure 8A) and quantified relatively to both matrix expression and cellular expression, according to previous described methods[31]. Agg matrix expression (Figure 8B) was higher than the control, in the NP area, both in the C+hMSCs and C+HAPSDF5+hMSCs, independently of the time point (C+hMSCs, day 7: (1.45 ± 0.41-fold), day 14 (1.54 ± 0.57-fold, **p<0.01) and day 21 (1.77 ± 0.34-fold); C+HAPSDF5+hMSCs day 7: (1.33 ± 0.32-fold), day 14 (1.58 ± 0.31-fold, **p<0.01) and day 21 (1.60 ± 0.22-fold); while in the C+HAPSDF5, matrix staining decreased at day 7 (0.86 ± 034-fold) and day 14 (1.00 ± 0.45) and slightly increased at day 21 (1.30 ± 0.12-fold). Similar trend was observed in the AF for C+hMSCs (day 7: (1.11 ± 1.01-fold), day 14 (1.10 ± 0.19-fold) and day 21 (1.19 ± 0.40-fold) and C+HAPSDF5+hMSCs day 7: (1.18 ± 0.87-fold), day 14 (1.07 ± 0.19-fold,) and day 21 (1.25 ± 0.61-fold), while in the C+HAPSDF5 was slightly higher at day 7 (1.12 ± 0.96-fold) and day 14 (1.03 ± 0.35-fold), decreasing at day 21 (0.98 ± 0.42-fold).

In what concerns to the pericellular staining of agg, the relative percentage of cells staining for agg was compared between treatments and during time and presented as a fold-change relatively to the cavity control (Figure 8C). Similar to what was observed in the matrix staining for the NP, the relative percentage of cells stained for agg was higher than the control in the groups containing hMSCs (C+hMSCs (day 7: 3.01 ± 5.3-fold, day 14: 1.70 ± 1.04-fold and day 21: 2.38 ± 1.34-fold, *p<0.05 relatively to the control, and **p<0.01 relatively to C+HAPSDF5 group; and C+HAPSDF5+hMSCs (day 7: 1.09 ± 3,61-fold, day 14: 1.41 ± 2.06-fold and day 21: 1.51 ± 1.83-fold)), while the in C+HAPSDF5 agg deposition decreased comparatively to the cavity control (day 7: 0.58 ± 3.32-fold, day 14: 0.86 ± 0.68-fold and day 21: 0.34 ± 1.32-fold). In the AF area, a higher expression relatively to the control was observed in the C+hMSCs, independently of the time point (day 7: 1.85 ± 2,67-fold, day 14: 1.70 ± 4.54-fold and day 21: 1.8 ± 1.12-fold), while in the C+HAPSDF5+hMSCS, an increase was observed at day 7 (2.07 ± 4.70-fold) and day 14 (1.06 ± 1.21-fold), but a slightly decrease can be noticed at day 21 (0.82 ± 0.92-fold). In the group congaing only the hydrogel (C+HAPSDF5), increasing

expression was observed at day 7 (1.13 ±4.22-fold), while decreasing in the following time points (day 14: 0.79 ± 1.20-fold and day 21: 0.63 ± 0.98-fold).

From these results, it was clear that the presence of hMSCs, had an impact on the increasing agg expression both in the tissue and in the pericellular area, namely in later time points, such as day 14 and day 21, as previously described[31]. Although, in the particular case of agg expression, no difference between both the groups (C+hMSCs and C+HAPSDF5+hMSCs) was observed, suggesting that this effect occurs regardless of the presence of chemoattract delivery system, and it is independent of the number of cells which migrated towards the tissue.



A

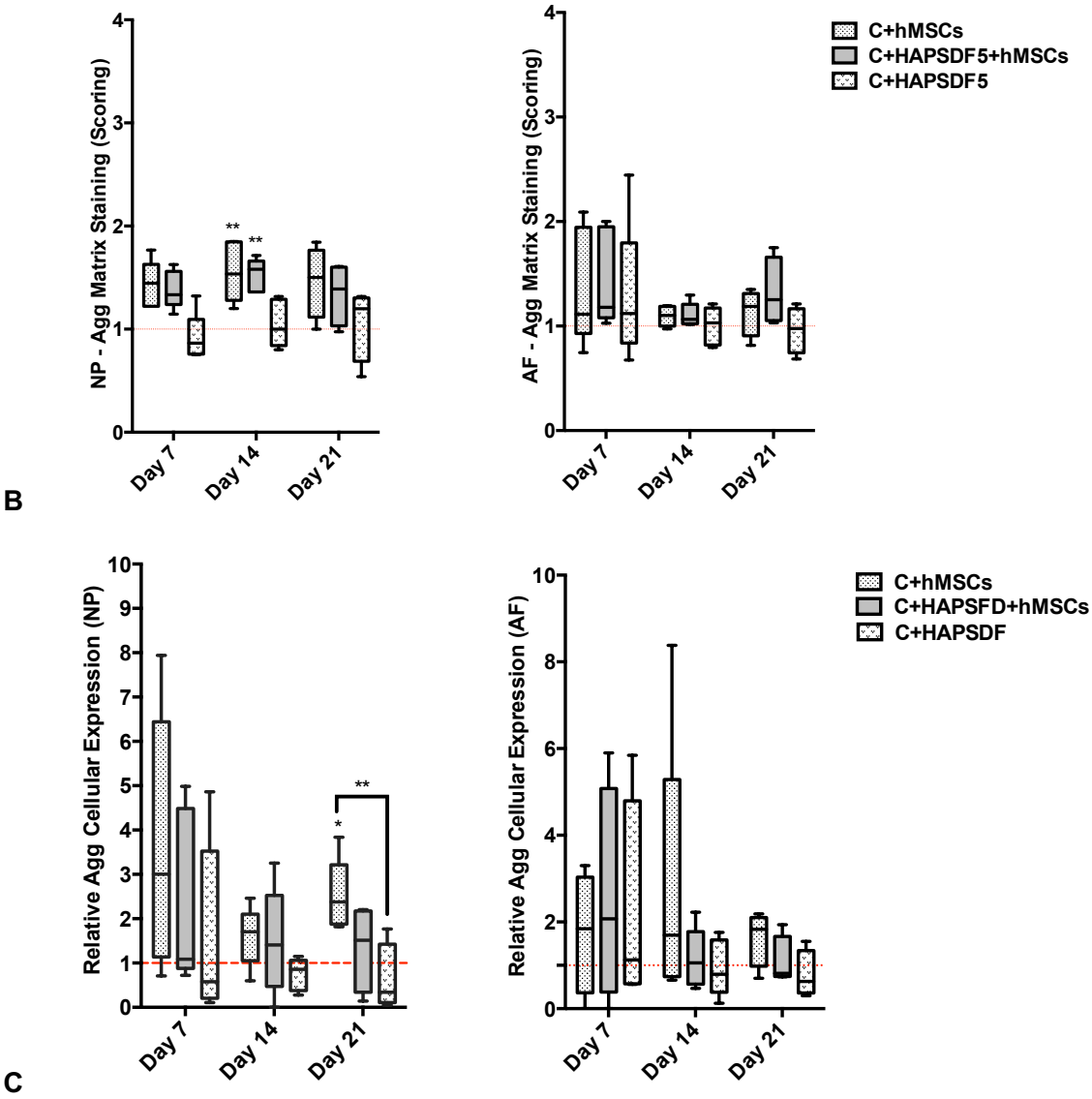


Figure 8 | Agg analysis in situ by IHC. Agg expression and distribution in the tissue was accessed by IHC in sagittal sections of the IVD. **A |** Representative sections of Agg expression both in the AF and NP of the different time points. **B |** Agg matrix staining quantification in the tissue. **C |** Agg pericellular staining quantification. Results are normalized by the cavity control and presented as box-and-whiskers plots (n=5-9). Statistical analysis was performed One-way ANOVA Kruskal-Wallis test. Statistical significance was considered when $p < 0.05$ (*), (**$p < 0.01$).

Growth factors involved in the HAPSDF5 effect

To further understand the role of HAPSDF5+hMSCs in the matrix remodelling, we have compared the levels of three different growth factors, known to be involved in matrix synthesis in the IVD and cartilage as previously identified by other works in the group[31], in both C+hMSCs versus C+HAPSDF5+hMSCs groups. For that purpose, the levels of TGF-β1, bFGF and GM-CSF were quantified by ELISA at different time points: day 7, 14 and 21, and correlated with a higher cell migration and matrix expression pattern observed.

TGF-β1 is known to be involved in col type II and agg metabolism of IVD cells[36]. Both at day 7 and day 14, TGF-β1 levels were higher in the C+HAPSDF5+hMSCs group, when compared to the C+hMSCs (day 7: 110.8 ± 99.34 pg/mL vs 97.23 ± 102.27 pg/mL; day 14: 86.50 ± 85.56 pg/mL vs 23.95 ± 70.52 pg/mL), while an opposite trend was observed at day 21, where the C+hMSC group presented a higher TGF-β1 concentration (79.69 ± 81.30 pg/mL) when compared to the C+HAPSDF5+hMSCs (35.75 ± 40.18 pg/mL) (Figure 9). These results, play along with those observed for col type II expression demonstrated by IF and WB at the same time points, suggesting that the observed effects, a higher expression of col type II at day 7 and 14 in the C+HAPSDF5+hMSCs group, might be correlated with a higher concentration of TGF-β1 in the media at the very same time points. On the other hand, a slight increase of TGF-β1 concentration can be observed in the group containing hMSCs only, at day 21, the time point at which we could also see a switch trend of col type II expression (higher in the C+hMSCs group).

bFGF has been associated with IVD cells proliferation[37,38] but also to hMSCs differentiation into NP-like cells[39]. In what regards to matrix synthesis, bFGF was described to increase cell biosynthetic activity and the deposition of col type II and glycosaminoglycans when supplemented in chondrocyte cultures[40], and to increase synthesis of sulfated proteoglycans by NP cells, during expansion[41], moreover, this growth factor seems to play a critical role in agg catabolism by controlling the activity of agg degrading enzymes in human articular cartilage[42]. Here in, the assessment of bFGF concentration in the culture media revealed that an increase in bFGF did not correlate with an increase in agg deposition. Although agg deposition increased similarly in both groups containing hMSCs, the levels of this growth factor were slightly different within this two groups. At day 7 an increase of bFGF can be observed in the C+HAPSDF5+hMSCs (46,45 ± 65,57 pg/mL) when comparing to the C+hMSCs (22.10 ± 22.90 pg/mL), while at day 14 and opposite trend is observed with higher variability (C+hMSCs: 150.30 ± 343.80 pg/mL and C+HAPSDF5+hMSCs 21.45 ± 178.08 pg/mL). At day 21 the levels slightly vary between the groups, C+hMSCs: 27.42 ± 90.21 pg/mL and C+HAPSDF5+hMSCs 50.00 ± 121.12 pg/mL.

The levels of GM-CSF, described to be involved in the agg and col type II synthesis by rat chondrocytes[43] has been previously identified IVD *ex vivo* organ cultures using in a growth

factor array[31], still, we could not detect it by ELISA in the C+hMSCs and C+HAPSDF5+hMSCs groups at any time point.

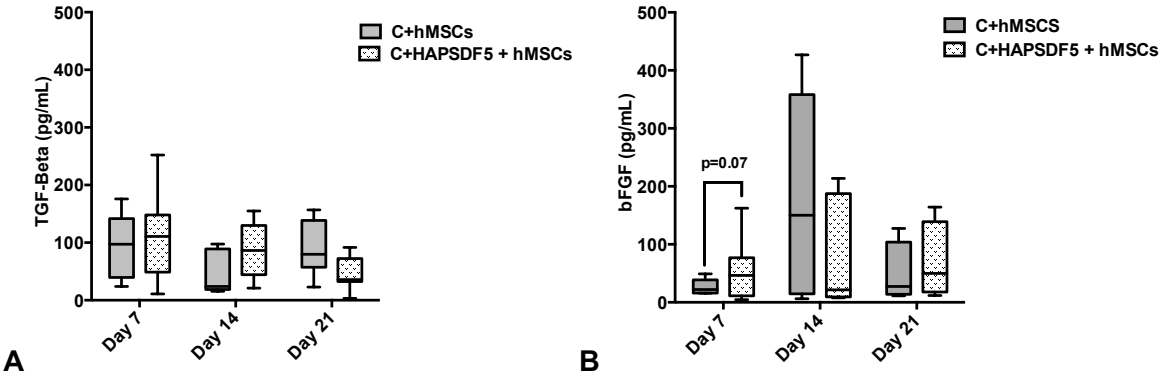


Figure 9 | Growth factors analysis in the IVD culture media. A | TGF-ß concentration in the culture along time. **B |** bFGF concentration in the culture along time. Statistical analysis was performed using Wilcoxon matched-pairs test.

DISCUSSION

This study aimed to clarify the potential of a chemoattractant delivery system (HAPSDF5), not only to enhance hMSCs recruitment to the disc[32], but also to accelerate or induce an early regenerative effect on IVD matrix remodelling. For that purpose, we have used a previously established *ex vivo* model of nucleotomy in bovine discs containing the CEPs[17]. *Ex vivo* models have been important tools in the study of IVD nature and new therapies[44]. Comparatively to *in vitro* studies, they offer a more complex environment that closely resembles the native tissue, thus providing the perfect and highly specialized ECM for cell maintenance[44]. The *ex vivo* culture of the discs with CEP prevents tissue swelling, maintaining the IVD integrity. Recently, using an *ex vivo* model with CEPs cultured for 21 days, we have demonstrated that hMSCs could migrate from the CEP towards the centre of the IVD and have a beneficial effect in what concerns to synthesis of the main IVD matrix components, col type II and agg[31], demonstrating the ability of these cells to migrate through the IVD tissue and moreover, the potential of the CEP as an alternative route for cell based therapies. Combining this results with the work of Henriknsson et al. that reported stem cell niches in the IVD surroundings and migrating routes towards the disc[24,25] and the establishment of a new transpedicular approach to reach the NP without AF damage by Vadalà et al.[45], new perspectives were opened in IVD degeneration treatment by giving rise to alternative ways of repopulating the IVD tissue, by stimulating the recruitment of stem cells from neighbour tissues or through cell-based therapies using the CEP as delivery route.

The release of chemokines during an injury and degeneration is a natural mechanism to recruit stem or immune cells to participate in the tissue repair. The IVD itself, has been demonstrated to secrete specific chemokines during the degenerative process, namely CCL5/RANTES[29,30]. Still, the endogenous repair mechanisms are sometimes insufficient to achieve a successful tissue regeneration. To enhance this effect, our team recently developed a chemoattractant delivery system, based on the combination of a thermoresponsible HA-based, containing a well-known chemoattractant, SDF-1[32]. Following the injection of this chemoattractant delivery system in the nucleotomized *ex vivo* IVDs, we were able to significantly trigger the migration of CEP-seeded hMSCs towards the lesion, 48h after treatment, therefore increasing the number of cells in the IVD tissue.

In this work, we hypothesized that by enhancing the hMSCs recruitment, using HAPSDF5, we would be able to improve tissue repair by increasing cell numbers, and induce an early or higher effect in the IVD matrix, therefore empowering the previously described effect of hMSCs alone. HAP hydrogel has been previously used in for IVD repopulation with hMSCs, by providing the matrix for cell transplantation and differentiation within the disc[17], while SDF-1 is recognized as one of the most potent chemoattractants for stem cells and has

been used, in other contexts to improve stem cell recruitment to injury sites[21-23]. Herein, SDF-1 was entrapped in the HA-based hydrogel (HAP) and injected in the cavity previously performed in the NP of each disc, previously to hMSCs seeding in the CEP. These discs were maintained in culture during 7, 14 and 21 days. Overall, independently of the time points, discs remained metabolically active, regardless of the groups (Figure | 3). SDF-1 release to the medium was significantly higher in the groups where SDF-1 was added exogenously (C+HAPSDF5+hMSCs and C+HAPSD5) 48h after treatment, corroborating our previous findings[32], while in the following time points, the values were residual and comparable to those found in the groups where no SDF-1 was added (Figure 2). This suggests that the SDF-1 effect might be very strict in time and confined to the first hours of culture. That would explain why hMSCs migration was higher in the C+HAPDF5+hMSCs in early time points when comparing to C+hMSCs; and very similar at day 21. We hypothesize that in such system, using an *ex vivo* model cultured and static conditions, the role of the chemoattractant delivery system can be masked during time. In this particular model cell migration was shown to occur independently of the presence of an enhancer and might be also triggered by the lesion/degradation itself[28]. This can represent a limitation of the model, or, in the other hand, might suggest that SDF-1 release should be sustained during time to attain a more efficient effect. SDF-1 release from the HAP delivery system has been previously shown*, in vitro*, to be sustained up to 7 days[32], although a burst release of 27% occurs in the first 6 hours. Though we can nor directly compare the *in vitro* as in an *ex vivo* scenario, SDF-1 release can be further accelerated due to the hydrogel degradation by the cells and secreted enzymes during the culture.

Cell migration was evaluated by tracking of previously CM-DiI-labeled hMSCs (Figure 5). The presence of HAPSDF5 not only recruited a much higher number of cells, particularly at day 14, but also a higher percentage of migration in deeper sections of the disc (Figure 5). These results were also in accordance with those observed relatively to the DNA content, in which a higher DNA content was found in the C+HAPSDF5+hMSCs at day 14 (Figure 4).

By enhancing hMSCs migration, we expected to accelerate or improve hMSCs regenerative effect in the nucleotomized discs, for that we have evaluated both col type II and agg expression in the IVD tissue at different time points. There is no doubt that the presence of hMSCs is the key factor in the observed effects, as the HAPSDF5 alone did not improve significantly the expression of these two matrix components. On the other hand, the higher recruitment of hMSCs at day 14, resulted in significantly increased col type II production (Figure 6), suggesting that HAPSDF5 through hMSCs recruitment can accelerate col type II synthesis in this model. In the particular case of agg, our results showed a significant higher matrix deposition of agg at day 14 in both hMSCs containing groups (C+hMSCs and C+HAPSDF5+hMSCs), however this occurred regardless of the presence of HAPSDF5 (Figure 8). In contrast, at day 21 the C+hMSCs group showed a significantly higher pericellular

agg expression comparatively to the cavity control and the hydrogel alone (C+HAPSDF5). In the light of the observed results, we hypothesize that a higher hMSC recruitment might empower col type II expression, but now agg.

The results observed have been further correlated with the presence of some growth factors, including TGF-β1 and bFGF, for their potential to enhance chondrogenesis of MSCs and ECM synthesis, as well to induce NP cell proliferation and aggrecan/col type II synthesis[46]. Free active TGF-β1 concentration was assessed in the IVD culture media. A higher concentration of this growth factor could be correlated with the results observed for col type II expression, as an increase of TGF-β1 was observed in the C+HAPSDF5+hMSCs group in early time points (day 7 and day 14), along with the higher expression of col type II observed in the same type points in the presence of HAPSDF5+hMSCs. bFGF was described to enhance the synthesis and accumulation of ECM in the metabolism of the growth plate or articular cartilage[47,48], in the IVD bFGF has been described to stimulate proteoglycans synthesis in a canine model[49] and to increase cell proliferation in discs of rats[41]. In this work, this factor did not seem to play a notable role in ECM depositions. A slight increase was observed in the C+HAPSDF5+hMSCs at day 7, although during the time course of the IVD cultures, this growth factor concentration in C+hMSCs and C+HAPSDF5+hMSCs groups was variable and did not correlate with a higher matrix deposition. Both TGF-β1 and bFGF have been associated to beneficial effects in cell proliferation and ECM synthesis, still other studies have also reported contradictory roles of this factors in the IVD. Wallach et al. reported an inhibitory effect of TGF-β1 on proteoglycans synthesis[50], while bFGF has also been suggested to have a catabolic role in disc homeostasis, by being upregulated in painful degenerated discs[51] or by inducing catabolism through the production of MMP-13 and inhibition of proteoglycans synthesis[52]. From these results, TGF-β1 can be suggested as one of the players in the beneficial effect observed by HAPSDF5-recruited hMSCs in this IVD *ex vivo* model. Still, we cannot exclude many other growth factors which might be involved, and that were not addressed in this study. Further to this, we weren't able to distinguished whether these growth factors were produced by the hMSCs or by the disc cells, stimulated by the presence of the hMSCs and the HAPSDF5. Even though we can associate a significantly higher migration of hMSCs, stimulated by HAPSDF5, with a significantly higher col type II expression at day 14, we should not also exclude the possible paracrine effect of hMSCs which remained in the CEP. To exclude this effect through migration-inhibition would be an achievement worth to be explored in the future. Additionally, *in vivo* evaluation of this chemoattractan-based system would add further insights on the real empowerment of HAPSDF5 in the enhancement of stem cell recruitment towards their potential in prevent or decelerate/revert IVD degeneration.

CONCLUSIONS

To repopulate the IVD with cells that could revert the degenerative process is an ongoing challenge in the IVD field. Our study has shown the potential of a chemoattractant delivery system containing SDF-1, HAPSDF5, not only in the enhancement of hMSCs recruitment towards the IVD, but also in accelerating their regenerative effects in the IVD ECM remodelling, namely Col II and agg production. This pro-regenerative effect appeared to be mediated by growth factos, namely TGF-b1 and bFGF at distinct time points.

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CHAPTER VII

CHAPTER VII – GENERAL DISCUSSION

GENERAL DISCUSSION

The IVD degeneration process has been unravelled along the past years, although there are still several challenges to face when thinking about new strategies of treatment. The IVD has a poor self-repair capacity and to stimulate its endogenous repair can be very demanding, as the success of any strategy will greatly depend on the degenerative state of the disc.

In this particular work, we aimed to stimulate cell migration towards the degenerative IVD. This approach has been explored in different contexts, as previously described in Chapter II, such as cardiac regeneration or bone fracture healing. Stem cell recruitment following damage occurs naturally in the human body and is mediated by the release of several chemoattractant molecules that direct cells to participate in tissue repair (Rennert et al. 2012). Though, this natural mechanism is sometimes insufficient to achieve total repair. This fact is particularly puzzling in the case of the IVD, due to its avascular nature and low cell content that hinder the release of molecules to attract the cells, as well as to the difficulty of cell access, that do not dispose of a vasculature to reach the targeted tissue. Multiple factors and events lead to the progress of disc degeneration. To develop a strategy that can restore the cellular levels and compensate for loss matrix, would be of major interest and would target two key factors of IVD biological function.

Motivated by ground-breaking achievements in the IVD field we hypothesized that repopulating the IVD with novel and healthy cells was a plausible working hypothesis. This is based on the existence of progenitor cells in areas surrounding the disc, that can be targeted and stimulated (Henriksson et al. 2009a, Henriksson et al. 2012, Sakai et al. 2012). Furthermore, Illien-Junger (Illien-Junger et al. 2012), demonstrated that IVDs cultured under degenerative conditions, attract hMSCs towards the disc tissue, suggesting that the IVD has the ability of recruiting cells during the degenerative process. Hence, our investigation work pursued three hypotheses: 1) whether MSCs could migrate from the CEP towards the IVD, stay in tissue and contribute to tissue regeneration; 2) how a chemoattractant delivery system for degenerated IVD could improve cell migration; and 3) how advantageous would it be to improve cell migration to IVD and enhance tissue regeneration.

In that regard, we have tested our hypothesis using an IVD *ex vivo* organ culture with discs from bovine tails, accordingly to the previously develop model by Peroglio et al. (Peroglio et al. 2013). Discs from bovine origin have been used in the development of explant disc cultures, due to their close anatomical size with human lumbar discs (14-22 mm diameter and 5-10 mm thick) and similar pressure (Alini et al. 2008). Moreover, bovine discs were described to have a similar proteoglycans synthesis rate, as well as a similar distribution of both agg and col type II , to human lumbar discs (Demers et al. 2004). Although *ex vivo* cultures do not fully

represent the circumstances of human discs in the spine, these models bridge the gap between *in vitro* and *in vivo* models, by providing the complexity of the native tissue and allowing the testing of several strategies for IVD regeneration, which would not be possible using human discs, due to limited access to healthy discs.

In this work, bovine discs containing the CEP were isolated. Through the CEP, the central part of the disc was accessed and a NP portion was removed mechanically. The maintenance of the CEP in the *ex vivo* organ culture might limit nutrients diffusion to the tissue in culture. Lee et al. compared the culture conditions of bovine discs with and without CEPs. Although there was reduced cell viability in the discs with CEP, their biosynthetic activity remained high comparatively to the free-swelling discs (Lee et al. 2006). Here discs were cultured up to 21 days, maintaining their metabolic activity. Moreover, the presence of the CEP in the explant discs prevents swelling of the *ex vivo* organ culture and maintains tissue integrity (Lee et al. 2006). Furthermore, the CEP also provided the route to access the NP. Commonly, the access to NP is made through the AF (Iatridis et al. 2013), but a novel transpedicular route to get to the NP through the CEP has been recently proposed (Vadala et al. 2013b).

Using the CEP as a novel route to access the NP is very promising for the delivery of cells and hydrogels, such as a chemoattractant delivery system to recruit endogenous cells. Vadàla et al. described for the first time a surgical technique to approach the NP. By introducing a 2-mm drill in the caudal vertebra through the pedicle and the inferior CEP, the authors were able to access the NP, in *ex vivo* ovine and human disc models (Vadala et al. 2013b), and later this approach was also applied *in vivo* using an ovine model (Vadala et al. 2013a). Nonetheless, this approach might be limited by CEP damage. The CEP has a key role in IVD nutrition and health, by providing nutrients to the NP and inner AF (Urban et al. 2004). Its lesion might induce a reduction in nutritional supply triggering the progression of the degenerative process. CEP damage has been previously described as a degenerative model, although using a larger CEP damage (3.5 mm) (Holm et al. 2004), when compared to the technique described by Vadàla. Additionally, the author further proposed the CEP tunnel sealing using scaffolds, thus avoiding matrix degradation or material extrusion, besides providing a way to delivery materials to the NP, with an intact AF (Vadala et al. 2015). Still, the degenerative stage of the IVD degeneration and the level of CEP calcification constitute a concern, and some precaution should be taken in applying this technique in such conditions.

Throughout this research project, an *ex vivo* model of health bovine discs was used. Though the mechanical damage can induce tissue loss and disorganization, the induced damage would still be surrounded by native healthy tissue. This healthy tissue would have a higher regenerative capacity when compared, for instance, to models which mimic IVD degeneration by enzymatic digestion of the matrix (Chan et al. 2013). Moreover, this model was cultured under static conditions without compression. These conditions do not represent

the normal biomechanical forces that a disc is daily subjected to and therefore they are a limitation in this study. Mechanical loading has a key role in the physiology and pathology of disc degeneration and might result in different biological responses; to apply the appropriate dynamic loading might benefit the synthetic activity and anabolic response of the disc (Wang et al. 2007), hence we cannot discard that our results could be affected if mechanical loading was applied.

The native IVD is known for being an hostile environment for the cells (Horner and Urban 2001), although disc cells have acquired the capacity to survive and function in such an environment, namely to survive under hypoxic conditions. Based on this, the establishment of hypoxic culture conditions are often applied when culturing disc cells (Gantenbein et al. 2014), explants (Teixeira et al. 2016b) or even when creating conditions for hMSCs differentiation into NP-like cell phenotype (Risbud et al. 2004). In our work, the IVD organ culture has been cultured under normoxic conditions, since its native structure was maintained during the culture, therefore providing similar parameters of oxygen and nutrients diffusion as those find *in vivo*.

In chapter IV, we have been able to demonstrate that hMSCs could migrate from the CEP towards the IVD (Pereira et al. 2016). hMSCs were seeded on the top of the CEP and allowed to migrate without any additional stimuli up to 21 days. These cells not only repopulated the IVD, but also gave a significant contribution to the synthesis of extracellular matrix. Both col type II and agg, two of the most abundant proteins in the disc, were highly expressed in the presence of hMSCs. These results are particular relevant as they are focused on their presence in the tissue, rather than on gene expression analysis. Col type II is responsible for the disc tensile strength (Antoniou et al. 1996) and its structural integrity in combination with proteoglycans is fundamental for the IVD function. Agg is a large proteoglycan which main function in the disc is to retain water, thus providing unique osmotic properties and the ability to withstand compressive loads (Sivan et al. 2014). Col type II and Agg degradation during degeneration has serious detrimental effects in disc function, so their reconstitution through the beneficial effects of migrating hMSCs is an important achievement in restoring the IVD function. In this work, besides looking to the matrix through the common techniques of histology, IF and IHC, we have further explored the IVD ultrastructure and the birefringence of the collagen fibbers. Both techniques have given new insights on how the damage and the ensuing treatment can impact on tissue ultrastructure and fibbers organization. Moreover, aiming to identify which growth factors could be involved in the effect of hMSCs, we have further analysed the IVD culture media for a vast array of growth factors, which was able to identify higher concentration of FGF-6, FGF-7, IGF-1R, IGFBP6, GM-CSF, PLGF, and SCF in the C+hMSCs group, while the levels of PDGF degreased relatively to the

control. In general, the identified factors could be correlated to the ECM metabolism and the presence of MSCs, suggesting that these cells might exert their function via paracrine effects.

In chapter V, the relevance of delivering SDF-1 using a hydrogel in hMSC recruitment enhancement was demonstrated. hMSCs were significantly triggered upon the injection of HAPSDF5 delivery system (Pereira et al. 2014), but not when SDF-1 was directly injected in the cavity of the disc. This suggested that SDF-1 effect might be impaired due to its rapid diffusion or degradation in the IVD tissue, a fact that can be circumvented when using a sustained delivery system. HAPSDF5 provided the creation of a chemotactic gradient in the first hours of culture that triggered hMSCs migration towards the tissue. This HA-based hydrogel not only provided an appropriate platform for chemokine release, but also offered unique characteristics for *in situ* delivery. HAP is a thermoreversible hydrogel that undergoes mild gelling formation mechanism, allowing its preparation and injection as a fluid solution at room temperature, becoming a gel at body temperature (D'Este et al. 2016). Moreover, HAP has been shown to maintain NP cells viability, morphology and re-differentiation towards NP-like phenotype upon expansion (Peroglio et al. 2012), as well as to induce hMSCs differentiation towards the disc phenotype (Peroglio et al. 2013). In chapter VI, the hydrogel by itself was shown not to have a notable effect on IVD matrix. Besides, following IVD histological processing, we were not able to visualize it in the cavity area. This problem has also been identified in the work of Li et al. (Li et al. 2016), that suggested the histological processing or loading as a possible explanation. Here we can exclude loading, but we cannot disregard its *in situ* degradation in the tissue or elimination during histology processing.

During this work, it also became clear that the responsiveness of stem cells is fundamental to achieve an effective outcome, when stimulating cell recruitment. Stem cell responsiveness to a chemotactic gradient is highly dependent on the expression of chemotactic receptors. In this particular work, the response to SDF-1 gradient upon release from the HAPSDF5 implies the expression of its receptors, CXCR4 and/or the rarer CXCR7. SDF-1/CXCR4 axis was shown to be critical for MSCs migration to lesion sites (Yellowley 2013). The expression of CXCR4 was not addressed in this work, although MSCs are known to highly express CXCR4 within the BM, although this expression is dramatically reduced upon cell expansion (Wynn et al. 2004). In the recruitment studies performed during this thesis, cells from different donors, with distinct ages, in passages 3-4 and comparable culture conditions were used. hMSCs from older donors were shown to have a lower response to the presence of SDF-1, when compared to hMSCs from younger donors (Pereira et al. 2014). The aging process leads to several changes in MSCs that can impair their potential not only to migrate, but also to participate in repair (Bustos et al. 2014). At the molecular level, *in vitro* aging of MSCs has shown a downregulation of several mRNAs related to the synthesis of chemokines, cytokines and their receptors, as for example SDF-1 and its receptor CXCR4, that

consequently affects their ability to migrate (Geißler et al. 2012). MSCs markers such as VCAM1, an important mediator of MSCs interaction with endothelial cells during the migration process, was show to decrease with aging (Turinetto et al. 2016). Therefore, the age of patients is a factor that should likewise be taken into consideration when applying a therapy that aims to stimulate stem cell migration. Additionally, when applying an SDF-1-based treatment aiming to recruit MSCs, we cannot discard that other cell types might be recruited, as CXCR4 is not exclusive of MCS, and has been described to be expressed in other cells, such as in EPCs (Ceradini et al. 2004) and T Lymphocytes (Kryczek et al. 2005).

In the chapter VI, using this model we evaluated how the recruitment of a higher number of cells could improve the pro-regenerative effect of hMSCs previously observed (Chapter III). For that purpose, hMSCs were tracked *ex vivo* and native ECM components of nucleotomized IVDs were analysed at distinct time points (7, 14 and 21). Col type II production appears to be accelerated and enhanced by the presence of HAPSDF5, as observed at day 14. This augment coincided with a higher number of hMSCs recruited, as well as a higher concentration of secreted TGF-ß, suggesting that this growth factor might be one of the key players involved. On the other hand, agg increased in both groups containing hMSCs, independently of the presence of HAPSDF5.

Although this model was appropriate to get proof of concept, in this particular case we could not observe strong improvement of HAPSDF5 in terms of IVD regenerative effects. This can partially be explained by a possible paracrine effect of hMSCs which do not migrated into the tissue, remaining in the CEP, but might still contribute to the observed effect, therefore diluting the beneficial effects of HAPSDF5. Other explanation can be related to the release of SDF-1, that was previously shown to occur in the first hours of culture (Pereira et al. 2014). In this work, SDF-1 could only be detected in the IVD culture media 48h after treatment, and only residual concentrations were found in the following days, suggesting that SDF-1 gradient action seems to be limited in time. To improve the sustained release of SDF-1 could be a strategy worth to explore in the future. In addition, this system should be tested *in vivo*.

The whole strategy here described proposes a novel vision to the treatment of degenerated IVD, that intends to prevent the progression of disc degeneration to the levels in which more invasive treatments could be the only available solution. The applicability of a therapy that targets the recruitment of stem cells through the injection a chemoattractant-delivering system using the CEP route should have a very tight window of action. This therapy should focus in early and mild degenerative stages, in which it would still be possible to revert the degenerative process by repopulating the disc with new cells and by stimulating the endogenous cells of the tissue. Moreover, it is also important to ensure that the main route for cell access to the disc, the CEP, is not completely blocked. Currently, most of the clinical trials are focused on therapies that target symptomatic patients with chronic low back pain, which

might not have their origin on disc degeneration. The eligible patients and the right timing to apply such a strategy will greatly depend on the evolution of imaging techniques and/or, on the discovery of new IVD degeneration biomarkers, which could identify early signs of degeneration. In the case of painful discs with herniation, it would be wiser to further apply immunomodulatory therapies, rather than only pro-regenerative therapies.

FUTURE PERSPECTIVES

This PhD thesis has contributed to the growing knowledge on stem cell recruitment for IVD degeneration, although there are still questions that remain to be answered and that would be very interesting to explore in the future.

One important aspect that was not addressed in this work and that would be fundamental to look at is what happens to hMSCs as they migrate through the IVD tissue. We have been able to identify a higher concentration of certain growth factors in the presence of hMSCs that are related with the ECM beneficial effects observed. Still, we were unable to understand if these factors were produced by the migrating hMSCs or by the disc cells, upon hMSCs paracrine stimuli. Adding to this, we have no indication on the possibility of these cells differentiating towards a disc-like phenotype in this particular setup. This could be addressed with an appropriate study of the expression of NP or AF-cells markers by human cells in the bovine IVD tissue.

In this work, the beneficial effect of hMSCs in the IVD regeneration was demonstrated. Still, there was always an intriguing question regarding the effect of the cells in the tissue: were we observing an effect of the migrating cells or a paracrine effect of the cells which remained in the CEP, or the effect of both? This is a limitation of this *ex vivo* model, which impairs the understanding of the real players in this effect. One possible strategy to address this issue would be inhibiting the migration through a CXCR4-antagonist. Further to this, it would be also very interesting to assess how ECM remodeling, by the increase of col type II and agg, would finally also improve the mechanical behavior of the disc. That would prove the restoration of the IVD biological function.

Finally, in the future the potential of the HAPSDF5 chemoattractant delivery system should be explored using an *in vivo* model. This will give an important answer to the relevance of such system in empowering cell recruitment to the disc upon damage/degeneration. This is currently being explored in collaboration with other colleagues. A pilot study has already been performed using a rat tail model of IVD degeneration, where HAPSDF5 was injected. Two strategies to address the potential of this system were tried, including by the injection of the chemoattractant delivery system HAPSDF5 alone. Using this strategy, we aimed not only verify how the presence of HAPSDF5 will favor endogenous repair, or if it would recruit transplanted

cells, by tracking cells bioluminescence on IVIS (Spectrum In Vivo Imaging system). So far, we were unable to track the cells, which might have to do to equipment resolution, but also due to direct migration to the lungs and signal loss after 24h. In the near future, cell migration by other techniques, such as histology and immunohistochemistry, should be attempted.

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